CHEMICAL PROFILING OF *Ganoderma lucidum* OF BATHINDA REGION

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In

Biosciences

BY

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CERTIFICATE

I declare that the dissertation entitled "CHEMICAL PROFILING OF Ganoderma lucidum OF BATHINDA REGION" has been prepared by me under the guidance of Dr. Sanjeev Kumar, Assistant Professor, Centre for Biosciences, School of Basic and Applied Sciences, Central University of Punjab. No part of this thesis has formed the basis for the award of any degree or fellowship previously.

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ABSTRACT

Chemical Profiling of *Ganoderma lucidum* of Bathinda Region.

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Ganoderma lucidum is a basidiomycete's fungus with numerous pharmacological properties. The important ingredients of Ganoderma lucidum are terpenoids and polysaccharides etc. which play momentous role in immunomodulating, anti-inflammatory, anti-cancer, anti-diabetic and anti-oxidative. Mechanism of anticancer is still unrevealed. Aim of the present study was to analyse phytochemical difference in the Ganoderma lucidum growing on different hosts in Malwa region. Biomolecules play an imperative role in growth and development. Stress condition remodels the physiology, morphology and development of plant. To combat with stress, plants evolve with time and synthesize secondary metabolites. Stress tolerance ability is generated by overexpression of isoenzyme, intracellular targeting of anti-oxidants and overexpression ability of anti-oxidative enzyme. Ganoderma lucidum was analysed for different parameters such as total sugars, reducing sugars, starch, proteins, phenols, antioxidant property and flavonoids by standard procedures which was collected in different stages of development on different hosts, such as Azadirachta, Acacia, Bauhinia, Melia, and Dalbergia spp. It manifests fungus-host relationship and amount of phytoconstituent synthesized. The biochemical estimation showed 38.1±0.0481 g/100g of total sugars, 19±5.925 g/100g of reducing sugars, 57.3±3.333 g/100g of starch, 42±4.2% of proteins, 9.7±0.066% of phenols, 86.31%±5.480 scavenging activity in term of % inhibition and 5.26 ±0.6 mg/g of flavonoids. Complete analysis shows that except flavonoids all phytochemicals content was exceptionally high. Terpenoids analysis showed variation within the different hosts. Ganoderic acid, which is most active anticancerous molecule showed variation within different hosts. It can be concluded from the preliminary studies that there are variations in the chemical constituents of GL with change in host which makes it a "chemovariant".

(Balraj Singh Gill) (Dr. Sanjeev Kumar)

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Balraj Singh Gill

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LIST OF ABBREVIATIONS

Sr. No.	Full form	Abbreviation
1.	Ganoderma lucidum	GL
2.	Thin layer chromatography	TLC
3.	High pressure liquid chromatography	HPLC
4.	Ganoderic acid	GA
5.	2,2-diphenyl-1-picrylhydrazyl	DPPH
6.	Retention time	RT
7.	Bovine serum albumin	BSA
8.	3,5-Dinitrosalicylic acid	DNSA
9.	Natural killer cells	NKS
10.	Interleukin	IL
11.	Ling Zhi-8	LZ-8
12.	Human immunodeficiency virus	HIV

CHAPTER I

INTRODUCTION

Ganoderma lucidum, small basidiomycete's fungus is mainly a filamentous fungus comprising of hyphae and reproducing sexually via the specialized cells called basidia, which grows on decaying logs and trees stumps. Ganoderma lucidum as a health and longevity promoter (Mahajna et al., 2008) is being used from ancient timesin China, Japan and other Asian countries, especially their fruiting bodies. Ganoderma lucidum, commonly known as Ling Zhi in China and Reishi in Japan is the scientific name for the species of red mushroom that was found in the wild habitats and is at present cultivated artificially. Ganoderma lucidum is soft, corky, flat and kidney shaped. Due to its medicinal value from ancient time, it was referred to as the "Herb of Spiritual Potency" and the "Ten Thousand Year Mushroom" in China. Nowadays, it is commonly referred to as the "King of the Herbs". Ganoderma contains nutrients that can maintain, promote or improve human health. Although, the fruiting bodies of Ganoderma lucidum are white which turn yellow before finally taking up a reddish brown colour, they appear in other colours such as red and yellow. Ganoderma is annual; its fruiting bodies are tough and can remain for a month. Once rare, but these days it is cultivated commercially for nutraceuticals purposes in China, Japan, Taiwan, Korea, and North America. Main species are G. applantum, G. tsugae, G. colossus, G. meredithiae etc., found mainly in China, Japan, Korea, Taiwan, eastern part of United States and northwest of Pacific region. In India, it is found in Kotdwar (Uttarkhand), Sirsa district (Haryana), Dehradun, New Delhi, Hoshiarpur (Punjab), Hisar (Haryana), Gujarat, Western Ghats. The basidiocarp, spores and mycelia contain different bioactive compounds such as triterpenoids, polysaccharides, proteins, flavonoids etc. which have been observed to play a pivotal role in various biological activities such as immunomodulating, anti-inflammatory, anti-tumor, anti-viral, anti-bacterial, anti-oxidative and anti-aging activity. In addition, Ganoderma lucidum mainly comprises of sugars, proteins, polysaccharides, peptides, amino acids, terpenoids, flavonoids, vitamins and alkaloids. Other activities exhibited by them include anti-carcinogenic, anti-oxidant (Jones et al., 2000), anti-mutagenic and anti-inflammatory (Lakshmi et al., 2003). Ganoderic acid B present in this fungus is known to inhibit HIV protease (Hsu et al., 2004**.** It is also observed to bring about aggregation of platelets and decrease in blood pressure attributed to the presence of polysaccharides and terpenes (Bao et al., 2002). Moreover, Ganoderma lucidum is also known to lower cholesterol level (Berger et al., 2004), in addition to acting as immunosuppressant and exhibiting mitogenic activity owing to the presence of proteins such as LZ -8 (Kohsuke et al., 1991). It is also seen to act as a parasite to different plants such as oil palm trees (Adaskaveg et al., 1986), coconut trees in South India and primarily on Dalbergia in Punjab and is known to enter into the root systems of many plants, such as oak, cedar, Pear etc. This fungus colonizes into the plant root system and damaging the plant within 10-20 years.

Ganoderma lucidum grows in and occupies mainly the Western Ghats and the adjoining areas, since here the conditions are favorable in addition to the easily available nutrients. Rainfall and temperature include other important factors for their growth. Bathinda is located in North West part of India, where rainfall is scanty accompanied by extreme climatic conditions with temperature rangingfrom -2° to 48°C. Such high differences in temperatures observed throughout the year may result in variation in composition of secondary metabolite to combat harsh conditions, as literature suggests that harsh condition may increase or decrease the level of phytochemicals. Thus, highlighting the fact that changes in production of different secondary metabolites is influenced to a large extent by temperature, humidity and rainfall. Production of terpenes etc. is negatively correlated with rainfall with some of the terpenes found only in summer season (Vallat et al., 2005). It is reported that Bathinda and its adjoining areas are highly prone to cancer (Singh et al., 2012) and abundance of this fungus is also noteworthy in this area. The anticancer activity of Ganoderma lucidum is attributed to the inhibition of signaling pathways involved with cell adhesion, proliferation, survival, invasion and degradation of the extracellular matrix (Lin et al., 2005; Jiang et al., 2004; Lin et al., 2003).

Environmental condition plays a crucial role in function and distribution of plants. It includes both biotic and abiotic factors that determine the morphology of the plant. G. lucidum growing in such conditions may be different from that grown under normal condition. Relationship between plants and other species depends on factors which determine the type of species growing on type of host. Depending on the climatic conditions, saprophytic nature of Ganoderma varies from region to region e.g. as in South India it is found in association with Coconut, palm etc. whereas in northern India, especially in Bathinda, it is mainly associated with Dalbergia, Acacia etc. with particular features that help them to sustain in this environment. This may be attributed to the changes in the chemical constituents of the species that occurred in order to enable them to adjust and sustain best in any given environment.

Hypothesis:

The impact that geographical distribution has on the general flora is undeniable owing to which the species available in Bathinda region is also thought to have different chemical composition. Moreover this location is known for the extreme climatic conditions, in terms of high temperature, low temperature, salinity, drought and heavy metals.The accumulation of secondary metabolites is also known to depend on the climate and the host. It is hypothesized that the species available here grow on different plant species and thus possessing different chemical profiles (Chemo-variants). Similarly, these chemo-variants are thought to be more active and potent for its anti-cancerous activity.

CHAPTER II

REVIEW OF LITERATURE

The literature and traditional knowledge pertaining to the Ganoderma lucidum was collected and categorized under different heads. The literature review carried out is complete and up to date to the best of our knowledge.

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2.1 Identification:

Ganoderma species are difficult to identify since the different species look alike. Although, the reliable morphological features include spore shape, size, colour and microanatomy of pilear crust, Ganoderma lucidum has two distinguishing parts: the mycelium and the fruiting body. The upper surface of the fruiting body, which is kidney or fan shaped, is shiny and dark red in addition to being smooth. It has yellow and white edge and which later becomes whitish growing edge eventually turning purple brown before turning to black with the passage of time. Mycelium is the basilar and white villiform, which supplies the nutrients needed for growth, while the fruiting body is like an umbrella form. Both the mycelium and fruiting body are edible and have various medicinal properties that can be used to prevent and ameliorate human diseases. Pileus may have shape that varies from reniform to sub-circular and convex to concave with sulcate zone and radial furrow present on their surface. The length of stipe may vary from short to long and the attachment of pileus to stipe varies from lateral to the central. Surface thickness of pileus differs from one layer to several layers. Colouration of spores varies from white to yellow and length of tube layer varies from short to long (Gottlieb et al., 1999).

2.2 Cultivation, use and *Ganoderma* products:

G. lucidum is being used as a medicine since the ancient times. Only a particular type may be found in a certain region since the climatic condition for the growth and survival varies from one species to other (Benzie et al., 2011). Owing to its importance, it is being cultivated around the world. Wood logs, sawdust and grains are utilized as substrate for artificial cultivation of G. lucidum (Adams et al., 2010). Substrate, nutrient medium and culture condition has strong influence on the growth of mycelia (Kim et al., 2002). In a recent study, it was also found that whey permeate can be used as an alternative growth medium in G. lucidum cultivation (Song et al., 2007). Polysaccharides production cultivation was observed to be maximum at a temperature of 30°C-32°C and pH condition of 4-5 (Yang et al., 1998). In two-stage cultivation of G. lucidum, addition of Phenobarbital, a P-450 inducer has been employed to enhance the production of total and individual ganoderic acid (Liang et al., 2010). On the contrary, the three stage light irradiation strategy was seen to be beneficial for the efficient production of Ganoderic acid (Zhang et al., 2008). Now-a-days, numerous Ganoderma products are available in the market.

2.3 Major Bioactive components:

Most mushroom contain 90% of water by weight, the remaining 10% constituting of 10-40% proteins, 2-8% carbohydrates, 3-32% fiber, 8-10% ash and some minerals such as calcium, iron zinc, copper etc. (Mizuno et al., 1995). In some studies, it was found that the non-volatile components consists of 1.8% ash, 26-28% carbohydrates, 3-5% fats, 7-8% proteins (Mau et al., 2001). Ganoderma also contains compounds belonging to the class of terpenoids, phenols, flavonoids, steroids, carbohydrates, low total fat content and high proportion of polyunsaturated fatty acids. High polyunsaturated fatty acid content relative to the total fatty acids make them crucial for health in term of nutritional values (Benzie et al., 2011). Triterpenes, polysaccharides and peptidoglycan are three main physiologically active components of G. lucidum (Boh et al., 2007).

2.3.1 Polysaccharides and peptidoglycans:

This fungus contains variety of high molecular weight polysaccharides, polyglycans and polysaccharides representing wide range of physiochemical properties (Zhou et al., 2009). Its fruiting bodies, spores and mycelia are the major sources of polysaccharides in fungus which differ from each other in their composition and molecular weight. Ganoderma lucidum polysaccharides (GL-PS) are reported to exhibit antitumor (Miyazaki et al., 1981), anti-inflammatory (Hong et al., 2004), immunostimulating effects (Gao et al., 2003). The active molecules can be extracted by hot water treatment followed by ethanol or methanol precipitation. Molecular weight of polysaccharides range from 4×10^5 to 1×10^6 Da in their primary structure and their solubility depends on temperature (Yuen et al., 2005). It was found while studying polysaccharides in Ganoderma lucidum that glucose is the major component (Wang et al., 2002). GL-PS are hetropolymer in structure containing mannose, galactose, fucose and xylose in different conformation i.e. 1-3, 1-4, 1-6 linked β and α-D substitution (Selbmann et al., 2002). Solubility characteristics and conformation in branching pattern determines anti-tumorigenic properties of polysaccharides (Benzie et al., 2011; Zeng et al., 2003). Refined polysaccharides preparations of G. lucidum has been marked for treatment of numerous diseases (Yue et al., 2008) like cancer and liver diseases. Various bioactive peptidoglycan (GLPG) have been isolated from G. lucidum which are known to possess antiviral activity of GLPG (Eo et al., 1999) and immunomodulating substances (GLIS) (Benzie et al., 2011). Polysaccharides extract inhibits the formation of Ras-induced transformed foci in an R6 embryo fibroblast cell line (Hsiao et al., 2004).

2.3.2 Triterpenes:

Terpenes such as menthol (monoterpene), β-carotene (tetraterpene) etc. are the naturally occurring compounds comprising of isoprene units. They are widely distributed among the plants, prokaryotes as well as in eukaryotes. Although, plants synthesize terpenes for their normal growth and development, latex and resins are the major products of this plant which play a pivotal role in providing resistance against many diseases. Numerous terpenoids exhibiting various therapeutic effects including anti-inflammatory and anti-tumorigenic activity have been isolated from plants. Triterpenes are the subclass of terpenes having basic skeleton of C-30 and molecular weight ranging from 400 to 600 kDa, possessing a chemical structure which is highly complex and oxidized (Zhou et al., 2009). In G. lucidum, triterpenes structure is based on lanostane (metabolite of lanosterol) biosynthesis which is based on cyclization of squalene (Hayashi et al., 2003). Terpenoids can be extracted with the help of solvents such as methanol, ethanol, acetone, chloroform, ether or by their mixture and the further separation can be carried out by normal and reverse-phase HPLC (Song et al., 2002). Ganoderic acid A and B are the first isolated triterpenes from G. lucidum (Benzie et al., 2011). Now-a-days, vast majority of triterpenes have been reported with unique features which include mainly ganoderic acid and lucidenic acid and to a lesser extent ganoderals, ganoderiols, ganodermic (Benzie et al., 2011; Zhou et al., 2009). G. lucidum is rich in terpenes which is responsible for giving the fungus medicinal values as well as the reason for imparting bitter taste to it. Terpenes content depends mainly on the growing stage of the plant. The profile of different triterpenes in Ganoderma lucidum can be used as a basis for differentiation of the medicinal fungus from other taxonomically related species. The triterpenes content can be used as a measure of quality of different samples (Chen et al., 2008).

2.3.3 Antioxidant property:

Electron acceptors such as molecular oxygen, react easily with free radicals to become radicals themselves, also referred as reactive oxygen species (ROS). The ROS include superoxide anions (O^{+2}), hydrogen peroxide (H_2O_2) and hydroxyl radicals (^{+}OH) (Mittler et al., 2004). Plants synthesize different types of free radical scavenging molecules such as flavonoids, anthocyanins, carotenoids, dietary glutathione, endogenous metabolites and many natural products having antioxidant property. Plant-derived antioxidants have function of singlet and triplet oxygen quenchers, peroxide decomposers, enzyme inhibitors (Awah et al., 2012). Cell damage and cell death is due to oxidation of biomolecules by formation of free radicals such as hydrogen peroxide, super oxides etc. These free radicals lead to ageing and various degenerative diseases (Manczak et al., 2006). Fruits, vegetables, tea, spices etc. are sources of antioxidants. In mushroom polysaccharides and phenols has been reported with antioxidant property (Cheung et al., 2003)

2.4 Biosynthesis of active molecule "Ganoderic acid".

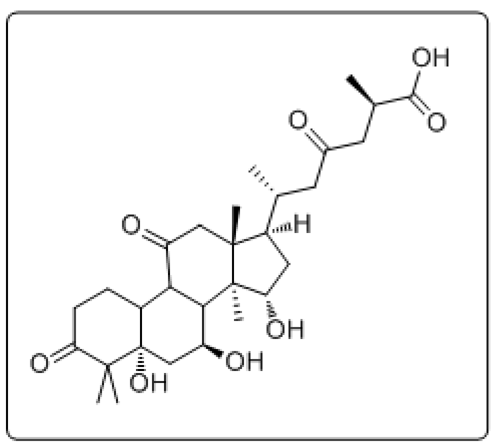


Figure 2.1: Chemical structure of Ganoderic acid A

The biosynthesis of ganoderic acid take place by isoprenoid biosynthetic pathways (Shi et al., 2010) starting with acetyl-coenzyme A and named as mevalonate pathway (MVP). First step involves the conversionof acetyl-coenzyme A into isopentenyl pyrophosphate (IPP) which are converted into higher order gernayl pyrophosphate (GPP), farnesyl pyrophosphate (FPP) and gernylgernyl pyrophosphate (GGPP) by the prenyl transferases. Intermediate self-condenses and used in alkylation reactions to give prenyl side chains of non terpenoids and ring to the basic structure to the terpenoids family. Finally different changes like oxidation, reduction, and isomerization gives the unique characters to the terpenoids.

2.5 Therapeutic applications:

Combined effect of benefit and toxicity results in their being used for various therapeutic interventions. From thousands years, it is being used as medicine to various problems related to health.

2.5.1 Anticancer properties:

Cancer is one of the major diseases responsible for claiming high mortality rate worldwide. It is a multi-factorial disease characterized by uncontrolled growth of cell subsequently leading to the formation of tumors. Despite the perpetual research being carried out it remains a challenge to solve the problem (Ferlay et al., 2010). Different types of bioactive molecules have been isolated from mushroom possessing anti-tumor activity including Ganoderma species (Zhang et al., 2009). Fruiting bodies, mycelia and spores are the main sources of chemical compounds. Triterpenes and polysaccharides are two main components that participate crucially in exhibiting antitumor activity (Mahajna et al., 2008). Mushrooms are effective in killing cancer cells as evidenced from the 58 samples of cancer cells tested with this species (Tomasi et al., 2004). Apoptosis and cell-cycle arrest is induced in humans and rodent tumor cells by G. lucidum (Gao et al., 2005) as suggested by the reports available on Human leukemia HL-60 (Liu et al., 2009), human hepatoma PLC/PRF/KB (Lin et al., 2003), human breast cancer (Martínez-Montemayor et al., 2011), human prostate cancer (Sliva et al., 2002), human colonic cancer (Thyagarajan et al., 2010), human cervix uteri tumors HeLa (Zhu et al., 2000) and human lung carcinoma (Cao et al., 2006). Progression of cell cycle at different level is regulated and arrested by G. lucidum, such as in lung at G1 stage, liver at G1/G2 stage, breast at G0/G1 stage. Extract of G. lucidum shows effect on various human cell lines, by suppressing the G1 stage of cell cycle and apoptosis. Caspases-7 and caspases-9 is activated by G. lucidum in breast cancer (Hu et al., 2002). It was also found that up regulation of cytochrome c and Bax triggers caspases-3 apoptosis in leukemia HL-60 cells (Liu et al., 2009).

2.5.2 Anti-inflammatory properties and anti-proliferative activity:

Triterpene extract of G. lucidum (GLT) shows decrease in secretion of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), nitric oxide (NO), prostaglandin E2 in RAW264.7 cells. GLT suppress the activity of NF-κB-DNA by inhibiting the AP-1 DNA binding activity and down-regulating expression of AP-1 subunit c-Jun. In vivo, it was demonstrated that GLT inhibits production of TNF-α and IL-6 in LPS-induced endotoxemic mice. Anti-proliferative activity is found in GLT by suppressing G0/G1–G2M stage which was mediated by down regulation of different cell cycle proteins such as cyclin D1,CDK4 and cyclin B1 (Dudhgaonkar et al., 2009).

2.5.3 Anti–Androgenic Osteoclastogenisis inhibitor:

Ganoderic acid inhibits prostate cancer cell growth and also inhibit osteoclastogenisis by inhibiting 5α reductase activity, which bind to the androgenic receptors (AR), (Johnson et al., 2010). In early prostate cancer, which is androgen-dependent, reducing the levels of circulating androgens to suppress AR/androgen signaling is an effective treatment. For example finasteride, 5α reductase inhibitor, which prevents conversion of testosterone to its more active form DHT in the prostate, is an effective drug for early prostate cancer. However, most prostate cancer patients eventually develop androgen-independent tumors that are resistant to this form of therapy. In many androgens independent prostate cancer cases, AR are highly expressed and hypersensitive to low level of androgens or even can be activated by non-androgens to induce tumor cell growth. Therefore, the strategy targeting the inactivation of AR function is particularly attractive to treat hormone refractory prostate tumors (Liu et al., 2009).

2.5.4 Anti-viral and anti- bacterial properties:

Anti-viral and anti- bacterial properties of plants against various kinds of infection led the researchers to work towards resistant and the mutant role of various molecules present in plants (Zhong et al., 2009). Isolation of various extracts in methanol, ethanol and chloroform showed inhibitory effect on herpes simplex virus (HSV-1), herpes simplex virus 2 (HSV-2) (Kim et al., 2000). Triterpenes of G. lucidum such as ganoderiols F and ganodermanontriol exhibits potent anti-HIV protease activity (Li et al., 2010) with 50% of inhibitory effect (El-Mekkawy et al., 1998).

2.5.5 Immunomodulation:

Resistance and sensitivity of body depends upon the functioning of immune system and there are some agents which enhance or suppress the system. Ganoderma lucidum acts as immunostimulant, inducing the cytokines and increasing the immune response (Lin, 2005; Sun et al., 2011). Bioactive compounds of G. lucidum such as polysaccharides and triterpenoids have proven to induce the formation of B- and T-lymphocytes and NK cells.

2.5.6 Anti- HIV activity:

HIV was isolated as an agent of immunodeficiency disease in 1983 (Barré-Sinoussi et al., 1983). Millions of people are suffering and dying with HIV or AIDS or AIDS related problems. Anti-HIV therapy includes nucleotides analogues such as 30'-azidothymidine (Kartikeyan et al., 2007). These analogues are potent inhibitors of reverse transcriptase (RT) and protease. It was known that natural plant products have got anti-HIV activity (Kartikeyan et al., 2007). Lucidenic acid O and lucidenic lactones were isolated from G. lucidum possessing the property to inhibit calf DNA polymerase and rat polymerase-1 (Gao et al., 2003). Ganoderiols F and ganodermanontriol isolated from G. lucidum is reported active against HIV-1 growth (Cooi et al., 2000). HIV protease was inhibited by ganoderic acid B (Hsu et al., 2004). It appears that there is a structural activity relationship for triterpenoids having anti - HIV activity (Min et al., 1999). Low molecular weight extract shows anti-HIV activity using XTT (2, 3-bis (2-methoxy-4nitro-5-sulfophenyl)-5-(phenyl amino) carbonyl) -2H-tetrazolium hydroxide) has cytopathic effect on human cell lines.

2.6 Proteins, peptides and amino acids:

Bioactive proteins from G. lucidum named as Ling Zhi-8 (LZ-8) have been isolated whichis a polypeptide having 110 amino acids with molecular mass of 12 kDa (Tanaka et al., 1989). LZ-8 has mitogenic (Kino et al., 1990) and immunosuppressive activity (Kohsuke et al., 1991). Amino acids found in G. lucidum exhibit antioxidant properties and are related with cancer and ageing (Sun et al., 2004). Polysaccharides–peptide complex is responsible for anti-oxidant effect. Polysaccharide peptide (GI-PP) shows anti-tumour and anti-angiogenesis effect in mice (Cao et al., 2006), which inhibits vascular endothelial cell proliferation. (Wang et al., 2006) isolated a protein having laccase activity and suppressor of human immunodeficiency virus (HIV)-1. Another protein with low sugar content (lectin) was extracted from mycelia (GLL-M) and (GLL-F) from fruiting bodies (Kawagishi et al., 1997) (Ye et al., 2002), have found three kinds of bioactive proteins (LZP-1, LZP-2, LZP-3) from fruiting body of G. lucidum having mitogenic activity. Cyclooctasulfur and oleic acid were isolated from G. lucidum having tendency to inhibit histamine release, which helps in treatment of inflammation, allergies etc. (Tasaka et al., 1988).

2.7 Human studies:

Ganoderma directly affect different steps involved in immune system. It has been used in androgen dependent as well asandrogen independent prostate cancer (Gao and Zhou, 2003). Polysaccharides rich extract of G. lucidum was given to 134 patients at different stages of cancer with dosage of 1800 mg/day for 12 weeks (Gao et al., 2003) and it was observed that cellular components of infected person enhanced to 80% i.e. interleukin (IL-2), IL-6, natural killer (NK) cell and interferon gamma.

Table 2.1: Therapeutic effects of bioactive compounds of proteins (Sanodiya et al., 2009)

Therapeutic effects	Bioactive compounds
Immunodulation: mitogenic activity, stimulation of immune response and complement system.	Protein LZ-8, β-D glucan, ganoderic acid
Anti-cancer, anti-tumour, chemo and radio prevention.	β-Dglucan, hetropolysaccharides lanosteroid
Anti HIV-1 and anti HIV-1 protease.	Triterpenoids, lucidenic acid, ganoderic acid
Anti-diabetic	Glycan, ganoderan B and D
Anti-inflammatory	Ganoderic acid C, 3-oxo-5α lanosta-8, 24 dien 21-oic acid
Anti-allergic	Ganoderic acid C and D
Anti-angiogenic activity	Ethanol extract (compound not reported)
Anti-herpetic	Acidic protein bound polysaccharides
Anti-oxidant	Chloroform extract (compound not reported)
Anti-mutagenic	Methanol extract
Cardiovascular and circulatory function	Powdered mycelium and water extract of mycelium (compound not reported)
Anti-proliferative activity	Ganoderic acid T
Anti-microbial, anti-viral, anti-fungal	Neutral protein bound polysaccharides; acidic protein bound polysaccharides, ganodermin.

2.8 Harmful effect:

Biotic and abiotic factors are responsible for vegetation of particular area. Many parts of plants are affected by various kinds of fungi, causing decay of trunk, branches and roots. Root decay by fungi is caused by Ganoderma spp. seen in different parts of India on different plants. The symptoms are yellowing of foliage, followed by gradual wilting and drying leading to top dying, ultimately complete death of the plant and wilt is the resultant of both root rotting and colonization of vascular system of stem by the fungus, which blocks water transport though fungal structures and fungal metabolites and its byproducts. Dalbergia, Azardirachta, Melia etc. are most affected plants in South West Punjab. Different kinds of fungi and their interaction with plants specify the time and extent of damage. Decay process in overall plants is slow process but in recent times, huge mortality is caused by G. lucidum in Prosopis and Acacia. Decay is confined to older woods in living plants and most affected parts of the plant are roots and butt region. Once affected the outer wood is immediately colonized by G. lucidum (Bhansali, 2012).

Keeping in view the present status of Ganoderma lucidum, the proposed study was carried out with following objectives

1. To study the chemical profiling with different assays and UHPLC.
2. To study the variation in the biomolecules in relation to specific host

CHAPTER III

MATERIAL AND METHODS

Initially the samples were collected from nearby places of Bathinda, Bir Talab, Chetak Park and Central University Campus. During collection of samples the stage of the fungus and host was recorded.

3.1Sample collection:

The morphology of the fungus was kept in mind while collecting the sample includes kidney or fan shaped fruiting body, reddish growing edge eventually turning purple brown and then ultimately black, small roundish type pores or tubes, when young white in colour which later turn brown.

3.2 Powder formation and storage:

Around 39 samples were collected from nearby regions of Bathinda as well as from adjoining regions of Bathinda, which was stored at RT (25°C). Main focus during sampling was on host on which fungus is growing and the stage at which fungus was present. The samples were fine powdered with liquid nitrogen and stored for further use**.** The collected samples were processed for following biochemical analysis**;**

3.3 Total Carbohydrates:

Extract preparation: 0.1g of oven dried plant material was homogenized in 5mL 80% hot ethanol and centrifuged at 5000 rpm for 5 minutes. Kept the supernatant, which is further used as an ethanol extract for estimation of total sugars, reducing sugars and residue for starch.

3.3.1 Estimation of total sugars:

Total sugars were estimated according to the method described by Yemm and Willis (Yemm et al., 1954). 0.5mL of ethanol extract was taken in a test tube to which 4mL chilled anthrone reagent was added and mixed gently. After adding glass beads, the test tubes were immediately placed in boiling water bath for 10 minutes. Cooled in ice bath and then the OD were read at 625nm. To determine its concentration, it was compared with standard curve of total sugars in glucose solution in distilled water (1mg/mL).

Table 3.1: Sample collection sites of Ganoderma lucidum:

Site of sample collection	Host for the sample	Stages of fungus	No. of samples collected
University campus	Azadirachta indica	Mature dried stage	1
	Melia azedarach	Young fruiting stage	1
	Pongamia pinnata	Young fruiting stage	1
	Pongamia pinnata	Young fruiting stage	1
Bir Talab, Bathinda	Dalbergia sisso	Young, mature dried stage	6
Dabwali, Haryana	Dalbergia sisso	Mature dried stage	1
	Melia azedarach	Young fruiting stage	1
	Acacia litotica	Young fruiting stage	1
	Prosopis spicegara	Mature dried stage	1
Chetak park, Bathinda	Acacia litotica	Mature dried stage	8
Thermal plant, Bathinda	Jasmine	Young fruiting stage	1
	Acacia litotica	Mature semi dried stage	2
Jassi Powali, Bathinda	Melia azedarach	Maturedriedstage	1
	Dalbergia sisso	Mature semi driedstage	1
	Azadirachta indica	Mature dried stage	1
	Soil	Young fruiting stage	1
Gundwana sahib, Bathinda	Azadirachta indica	Mature dried stage	1
Talwandi sabbo, Bathinda	Azadirachta indica	Mature dried stage	2
	Dalbergia sisso	Mature dried stage	1
	Melia azedarach	Mature Dried stage	1
Pathankot, Punjab	Bauhinia spp.	Mature Dried stage	2
	Dalbergia sisso	Mature Dried stage	1
Dharmshala (H.P)	Jacuranda spp.	Semi dried stage	2
	Dalbergia sisso	Dried	2

3.3.2 Estimation of reducing sugars:

Reducing sugars estimation was done by Dinitrosalicyclic acid method by (Odunfa, 1985). 1mL of DNSA (Dinitrosalicyclic acid) was mixed with 200µL of 80% hot ethanol extract and boiled for 12 minutes. Cooled it at room temperature after adding 2mL of distilled water. Read at 560 nm spectrophotometrically compared with standard curve of reducing sugars. Final quantity of reducing sugars was expressed in g/100g of dry weight.

3.3.3 Estimation of starch:

200µL of ethanol extract was mixed with 3mL distilled water and 4mL 52% perchloric acid. Kept in ice bath for 20 minutes. Now 200µL of this extract was mixed with 2mL distilled water and 3mL of anthrone reagent. Kept in ice bath for 5 minutes. Boiled the sample mixture at 100°C for 8 minutes. Cooled to room temperature. Read at 630nm and compared with the standard curve of glucose for determining the concentration of starch in the sample (McCready et al., 1950).

3.4 Estimation of total proteins:

To 0.1 g of powdered sample, 1mL of distilled water was added. Homogenized for 10 minutes and centrifuged at 6000 rpm for 5 minutes. To 100µL of supernatant; 100µL of 1M NaOH was added followed by 3mL Bradford reagent to it. Mixed well, and read at 595 nm and compared with standard curve for determination of concentration of proteins in the sample (BSA is taken as standard 100µg/100mL) (Kruger, 2002).

3.5 Estimation of phenolic compounds:

To 0.1g of powdered sample, 1mL of methanol was added. Homogenized for 10 minutes and centrifuged for 6000 rpm for 5 minutes. To 200µL of supernant, 1mL of Sodium carbonate and 1mL of water was added. Mixed well and add 0.1 mL Folin Ciocalteau reagent (2N). Kept it for 1hr, read the OD at 765 nm and compared with standard curve for determination of phenols in the sample (Gallic acid 10mg/10mL used as standard) (Ghimeray et al., 2010).

3.6 Anti-oxidant activity in term of % inhibition (DPPH assay):

2, 2-diphenyl-1-picrylhydrazyl is a dark colour crystalline powder having free radical molecules and scavenges other radicals, therefore the rate reduction of reaction on DPPH used as indicator for radical nature. DPPH radical shows violet colour which become colourless or yellow when neutralized. This property allows visual monitoring of the reaction and the number of initial radicals can be counted from the change in the optical absorption at 520 nm. Free radicals scavenging property was measured by 2, 2 –diphenyl-1-picryl-hydrazyl. Reaction mixture (4mL) consisted of 2mL of DPPH in methanol (0.4mM), 2mL of extract and 2mL of methanol. Incubated for 30 min in the dark. Read the OD at 517 nm (BHT-butylated hydroxyl toulene is used as standard) (McCune et al., 2003).

3.7 Estimation of flavonoids:

To 0.1g of powdered sample, 2mL of 80% methanol was added. Homogenized for 10 minutes and centrifuged for 5 minutes at 6000 rpm. Kept the supernatant for further processing. To 0.3 mL of methanol extract, 0.06mL of 10% aluminum chloride was added. Mixed and gently added 0.06 mL of potassium acetate. Further to this 3mL of 80% of methanol was added. It was vortexed for 5 minutes and read the OD at 415 nm. The final content was measured with standard curve of flavonoids (chlorogenic acid 15mg/mL) (Ghimeray et al., 2010).

3.8 Pooled sample analysis:

Table 3.2: Pooling of host wise samples of GL

Name of the host	**Sample number**	**Stage of the fungus**
Acacia GL	15,16,17, 20,21,22,25,36	Growing stage, dried mature stage
Bauhinia GL	2, 3, 4	Dried mature stage
Melia GL	26, 28,35,38	Growing,dried mature stage
Azadirachta GL	1, 29, 30	Dried mature stage
Dalbergia GL	7, 8, 9,10,11,27,32,33,37	Growing stage, dried mature stage

The samples were pooled on the basis of host specificity for TLC and UHPLC analysis. In this pooling 5 sample were prepared from 39 samples after pooling.

3.9Terpenoids analysis using Thin Layer Chromatography (TLC):

Pooled samples were analyzed on TLC for the presence of terpenoids.

Terpenoids extraction for TLC: 1 g of dried powdered sample was added to 10 mL of ethanol and kept in sonicator for 1 hour. Transferred the supernatant to a fresh beaker and added 10 mL of ethanol to the residue left in a beaker and kept in a sonicator for another 1hour. Pooled the supernatant and concentrated in a vaccum evaporator at 70°C. This concentrated extract was used for running the TLC using silica gel plates as stationary phase and chloroform: methanol (10:1) as mobile phase. The plates were developed after drying by spraying an iodine solution (Aryantha et al., 2002).

3.10 Polysaccharides analysis using Thin Layer Chromatography (TLC):

Polysaccharides extraction for TLC: 1g of powdered fruiting body was soaked in 30 mL of hot distilled water (90-95°C) for 48 hours. The extract was then hydrolyzed with sulphuric acid 1M at 100°C for 8-16 hours then neutralized with barium hydroxide. The precipitation of barium sulphate was then removed after centrifugation at 6000 rpm for 5minutes. The supernatant was concentrated by evaporating at 70°C on water bath before running TLC.

TLC: Polysaccharides detection was done by spotting the extract on silica gel plate as stationary phase and n-butanol: acetic acid: ether: water (9:6:3:1) as mobile phase. Observe the spots by spraying concentrated sulphuric acid (Aryantha et al., 2002).

3.11 High Pressure Liquid Chromatography (HPLC):

Ganoderic lucidum has different bioactive ingredients of lanostane type triterpenes such as ganoderic acid and lucidenic acid. HPLC is used for complete separation and identification of triterpenes (Shiao et al., 1989) with minute amount of material (Mutlib et al., 1998). For chromatographic separation, temperature is an important factor affecting selectively, resolution and column efficiency (Castells et al., 2000a).

Retention temperature varies 15-55°C. In this method, powdered sample i.e. 100 g is mixed with 70% methanol. Kept in extractor for 6 days. Evaporated methanol extract to near dryness. Dissolved the extract in 500 mL water. After adding water, it was removed (water) with chloroform. Chloroform extract was subsequently dried under vacuum. Sticky paste obtained. It was stored as stock. Stock solution was dissolved i.e. 100 mg in 1mL methanol. Store at -20°C, for further processing.

The biochemical analysis was done in triplicate and subjected to statistical analysis.

Statistical analysis:

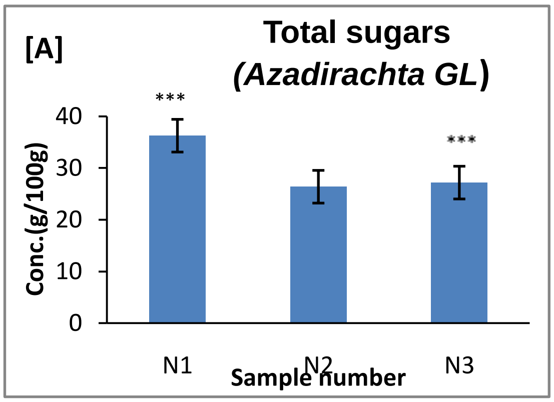
Results are expressed as mean standard deviation of triplicate experiments. Data were subjected to one way analysis of variance (ANOVA) and significant differences of mean were determined statistically using Tukey's test with stat software (Sigma plot). Level of significance was measured at $p<0.001$, $p<0.005$, $p<0.05$.

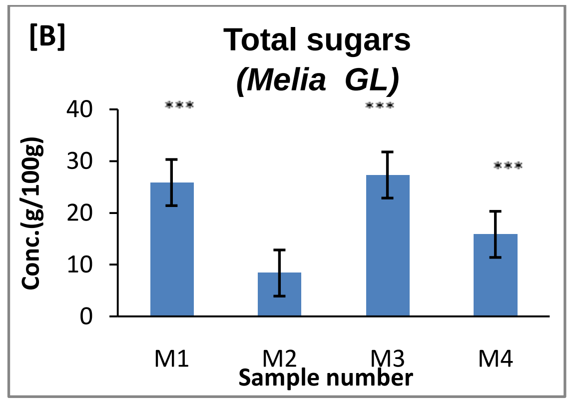
CHAPTER IV

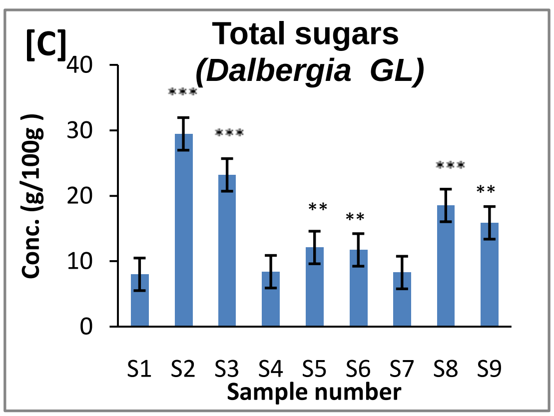
RESULTS

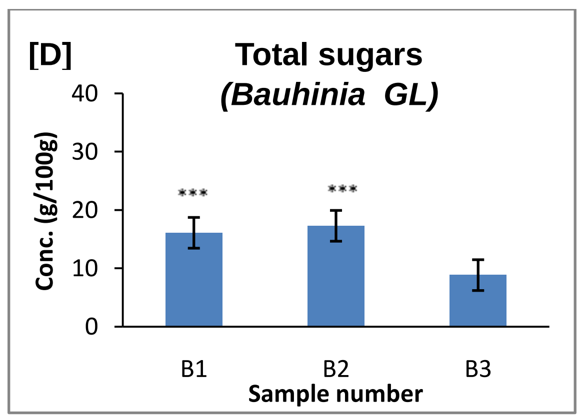
The collected samples were analyzed for different biochemical parameters such as total sugars, reducing sugars, starch, phenols, proteins, antioxidant property and flavonoids activity. The results obtained in the course of study are presented graphically as well as with suitable explanation.

4.1Total sugars content:









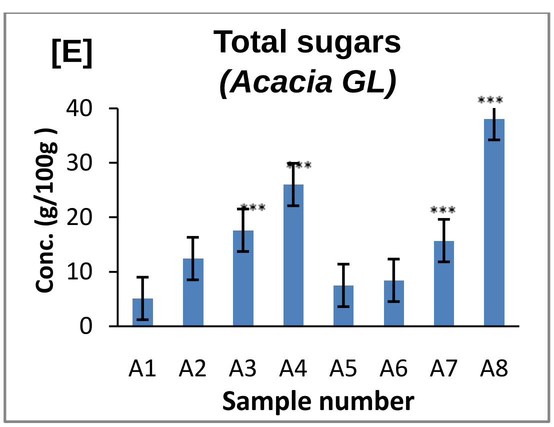


Figure 4.1 Total sugars content in [A] in Azadirachta GL [B] Melia GL, [C] Dalbergia GL, [D] Bauhinia GL, [E] Acacia GL.

Level of significance was measured at

*** represent ($P<0.001$),

** represent ($P<0.005$)

4.1 Total sugars (TS):

Estimation of total sugars was done for three Azadirachta GL samples i.e. N1, N2, and N3. Among these samples, N1 showed highest TS content (30g/100g). This may be due to growing stage (fresh samples) whereas N2 and N3 were in mature dried stage.

When compared the mean value of different samples of Azadirachta GL, N1 TS content was significantly higher ($P<0.001$) than N2 and N3, at the same time no significant difference was observed in the TS content of N2 and N3 (Fig 4.1A).

In case of Melia GL, four samples were analyzed, of which, M3 showed highest TS content (27.33g/100g). When compared the mean values, no significant difference was observed between M1 (25.87g/100g) and M3 (27.33g/100g) but both the samples had significantly higher content ($P<0.001$) than M2 (8.4g/100g) and M4 (15.87g/100g) (Fig 4.1B)

In case of Dalbergia GL, nine samples were analyzed, of which, S2 had highest TS content (29.47g/100g) followed by S3 (23.2g/100g) and S8 (18.53g/100g), which were significantly different ($P< 0.001$) from each other whereas S1, S4, S7 samples had lowest TS content (8, 8.4 and 8.27g/100g) respectively. These variations among the samples is apparently due to difference in the fresh and dried form i.e. S1, S4 and S7 were in dried form S5, S6 and S9 were in semi-dried form, and S1, S4, S7 were completely dried. When we compared mean values of different samples TS content of S5, S6 and S1, S4, S7 was at par (Fig 4.1C).

In case of Bauhinia GL, total sugars were estimated in three samples and no significant difference was observed in B1 and B2 but their content was significantly highest (17.33 g/100g) as compared to B3 ($P<0.001$)(Fig 4.1D).

In case of Acacia GL, eight samples were subjected to TS analysis; large variations were seen in these samples. Highest content of 38.1g/100g was observed in A8 followed by A4 (25g) and A3 (17.6g), A7 (5.7g/100g), which were significantly different from each other whereas A1, A5 and A6 showed low TS content. Here also the variation in TS content existed in fresh and dried samples (Fig 4.1E). Azadirachta GL showed the highest TS content while large variations were seen in Acacia GL. The TS content was maximum in Acacia GL (38.1g/100g) followed by Azadirachta GL

(36.27g/100g), Bauhinia GL (29.47g/100g), Melia GL (27.33g/100g) and Dalbergia GL (17.33g/100g).

4.2 Reducing sugars (RS):

Estimation of reducing sugars was carried out in for three samples of Azadirachta GL. Sample N1 was in the growing stage and had a highest RS content (19 g/100g) as compared to other two samples N2 and N3 (2.8, 3.4 g/100g) respectively (Fig 4.2A), which were collected as dried samples. When compared the mean values of different samples of Azadirachta GL, N1 RS content was significantly higher ($P<0.001$) than N2 and N3, at the same time no significant difference was observed in the RS content of N2 and N3.

In case of Melia GL, four samples were analyzed, of which M3 (fresh fruiting body) showed highest RS content (12g/100g) as compared to other samples, also it was significantly higher ($P<0.001$) than M1, M2 and M4 (3, 2.6, 3.2g/100g), respectively. Further M1 and M2 did not show any significant difference. Again it revealed that fresh fruiting bodies had highest RS content compared to dried ones (Fig 4.2B).

While analyzing nine samples of Dalbergia GL, the highest content of RS was observed in S8 and S2 (6.6g/100g and 6g/100g) followed by S9 (5.1g/100g) having a significant difference among them ($P<0.001$). RS content in host specific GL was exceptionally low in remaining samples. Also Ganoderma growing on this host plant did not have even comparable RS content than previous hosts Azadirachta and Acacia (Fig 4.2C).

Among three samples of Bauhinia GL, B2 had highest content (6.8g/100g) than other two samples, which were collected and dried. RS content in this sample was significantly higher than other two samples B1 and B3 (4.2 and 2.4g/100g) ($P<0.001$). Againthe fresh fruiting body supported the highest content of RS over dried ones (Fig 4.2D).

In case of Acacia GL, A8 showed highest RS content (11.9g/100g), which was significantly higher ($P<0.001$) than the remaining dried samples. Apparently no significant difference was seen in the samples showing low content (Fig 4.2E).

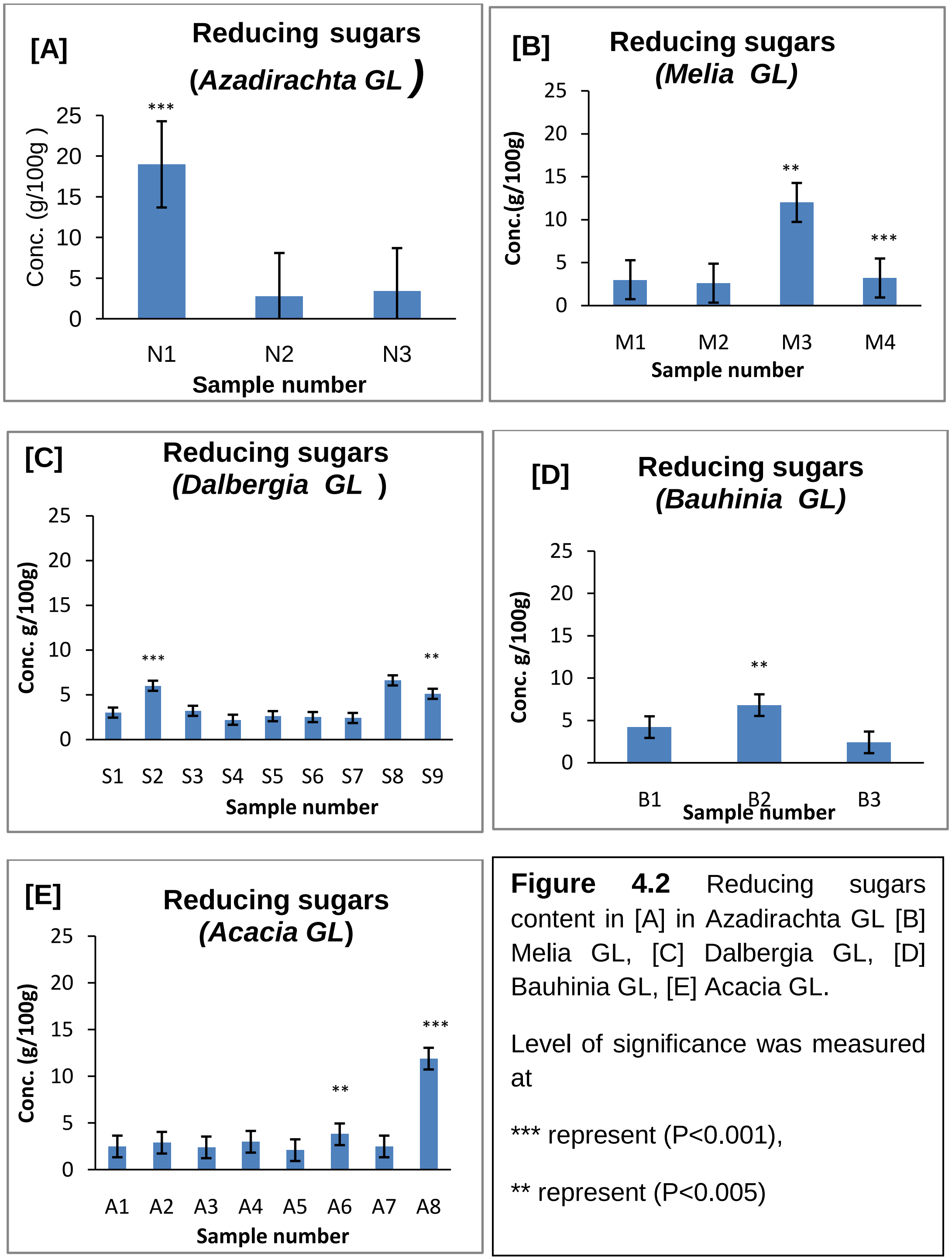


Figure 4.2 Reducing sugars content in [A] in Azadirachta GL [B] Melia GL, [C] Dalbergia GL, [D] Bauhinia GL, [E] Acacia GL.

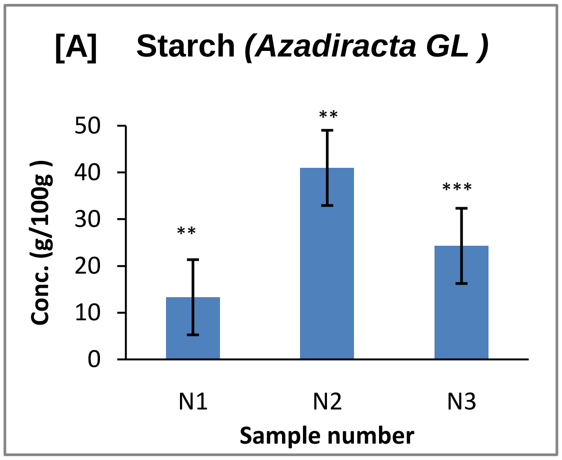
Level of significance was measured at

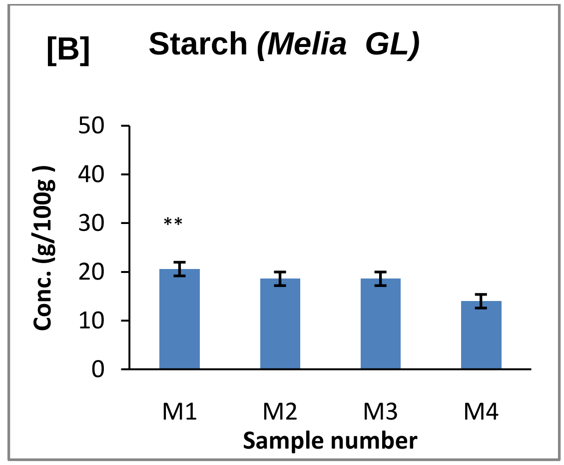
*** represent ($P<0.001$),

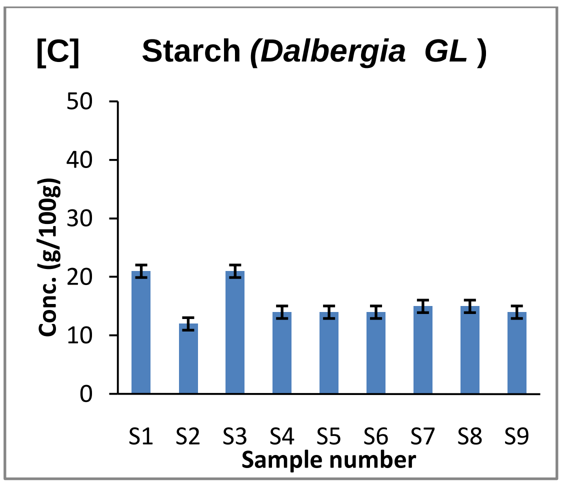
** represent ($P<0.005$)

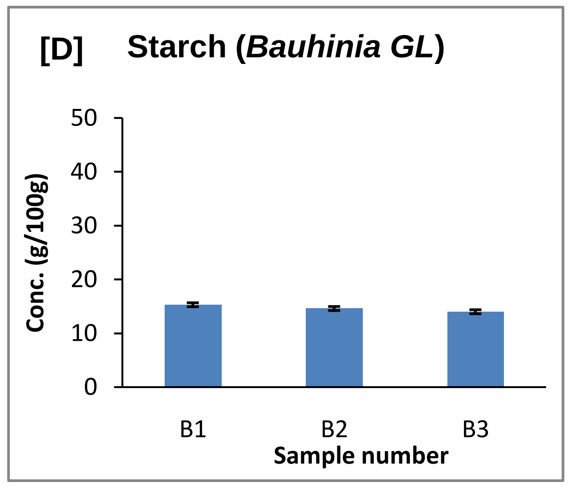
It can be concluded here that this fungus is growing on its all host plants but here are differences in RS content in host specific GL.

4.3 Starch content:









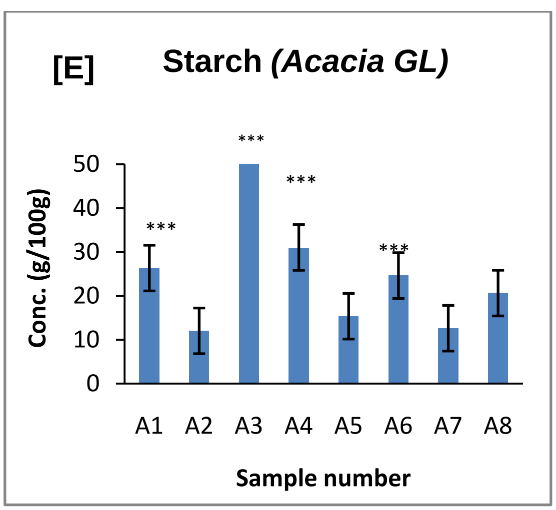


Figure 4.3 Starch content in [A] in Azadirachta GL [B] Melia GL, [C] Dalbergia GL, [D] Bauhinia GL, [E] Acacia GL.

Level of significance was measured at

*** represent (P<0.001),

** represent (P<0.005)

In Azadirachta GL, highest starch content was observed in N2 (41g/100g) followed up by N3 (24.3g/100gm) and N1 (13.3g/100gm), respectively. Significant differences were observed in N2, N3 and N1 (P<0.001) (Fig 4.3 A).

Starch content in four samples of Melia GL showed almost similar pattern. It indicated that developmental stage did not have any influence on the starch content of the fungus. Starch content in the entire samples was almost same. Different stages did not show any effect on the starch content. Significant differences were observed between sample M1 and M2 ($P<0.050$), while other samples M2, M3 and M4 were at par (Fig 4.3 B).

In Dalbergia GL, almost all the samples showed similiar results. Mean value of samples did not show any significant differences except S1 and S3, which were significantly different from rest of the samples (Fig 4.3 C).   .

Starch content in Bauhinia GL showed almost similar pattern in term of their quantity. No significant differences were observed among B1, B2 and B3 (Fig 4.3 D).

Starch content showed variation in Acacia GL, it was recorded highest in sample A3 (57g/100g) followed by A4 (31g/100gm) which was significantly different at $P<0.001$. While A2 (12g) and A7 (12.6g) had lowest content without any significant difference. Here also A3, A4, A6 and A8 were in the growing stage, this may be the reason that they had higher content, whereas A1, A2, A5 and A7 were in dried form with least starch content (Fig 4.3 E).

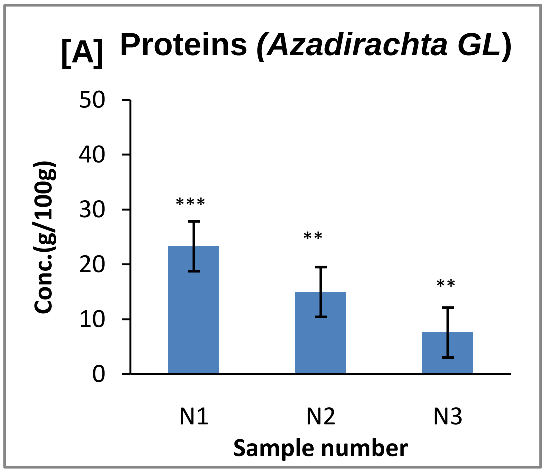
Melia GL, Dalbergia GL and Bauhinia GL did not have much variation in starch content, while Azadirachta GL and Acacia GL differ significantly. A3 (57) and N2 (41) g/100g were highest in all starch content while A2 (12), A7 (12.6) and N1 (13.3) g/100g were lowest in starch amount. It can be concluded that host plays an important role in Ganoderma starch content, while some host specific GL has influence on developmental stage.

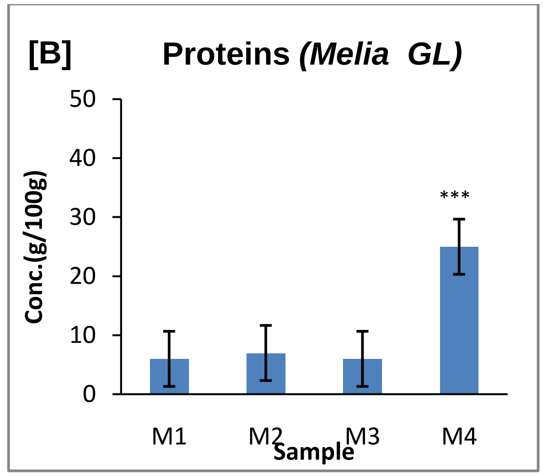
4.4 Proteins content:

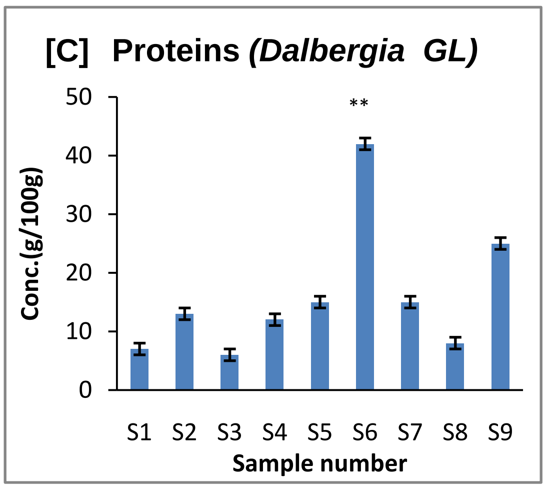
Significant differences ($P<0.001$) were observed in three samples of Azadirachta GL. Highest protein content was noticed in N1 (23.33g) followed by N2 (15g) and N3 (7.6 g/100g) (Fig 4.4 A).

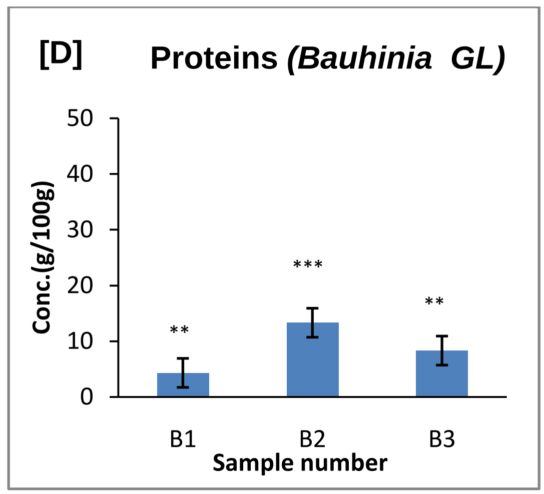
In case of Melia GL, highest content was found in growing stage of M4 (25g/100g), while M1 (semi dried), M2 (dried) and M3 (fruiting bodies) had lowest content of 6, 7 and 6(g/100g). Protein in M4 content was significantly higher

(P<0.001) than rest of the samples, whereas, no significant difference was observed in the protein content of other samples (Fig 4.4 B).









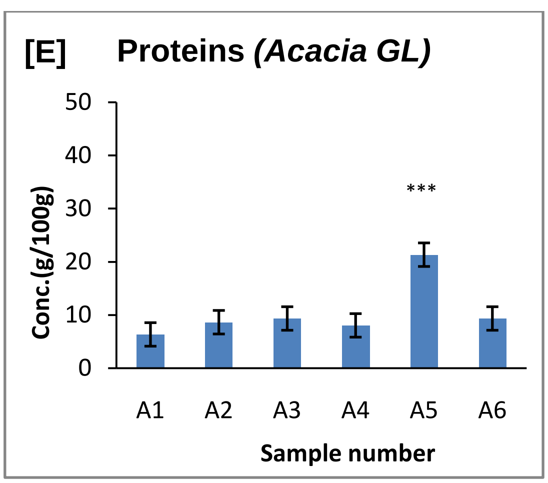


Figure 4.4 Proteins content in [A] in Azadirachta GL [B] Melia GL, [C] Dalbergia GL, [D] Bauhinia GL, [E] Acacia GL.

Level of significance was measured at

*** represent (P<0.001),

** represent (P<0.005)

Estimation of protein was done for nine samples in Dalbergia GL, in which S6 had highest content of 42g/100g, which was in semi-dried stage, whereas growing stage S2, S3, S5, S8, S9 had somewhat intermediate content. Dried samples S1 and S4 showed lowest protein content amongst all. When compared the mean value of different samples of Dalbergia GL, no significant difference was observed in the protein content (Fig 4.4C)

In case of Bauhinia GL, highest content was observed in B2 (13.3g) (dried sample) whereas B1 (4.3) and B3 (8.3g/100g) in fresh samples had low content of protein. There was significant difference between B2 and B1 ($P<0.001$) (Fig 4.4 D). Overall, minimum protein content was observed in Bauhinia GL.

In Acacia GL, highest protein content was observed in A5 (21.3g/100gm), whereas significantly less content was observed in remaining five samples. Also these samples did not show any significant difference in their protein content ($P<0.001$) (Fig 4.4 E).

4.5 Phenolics content:

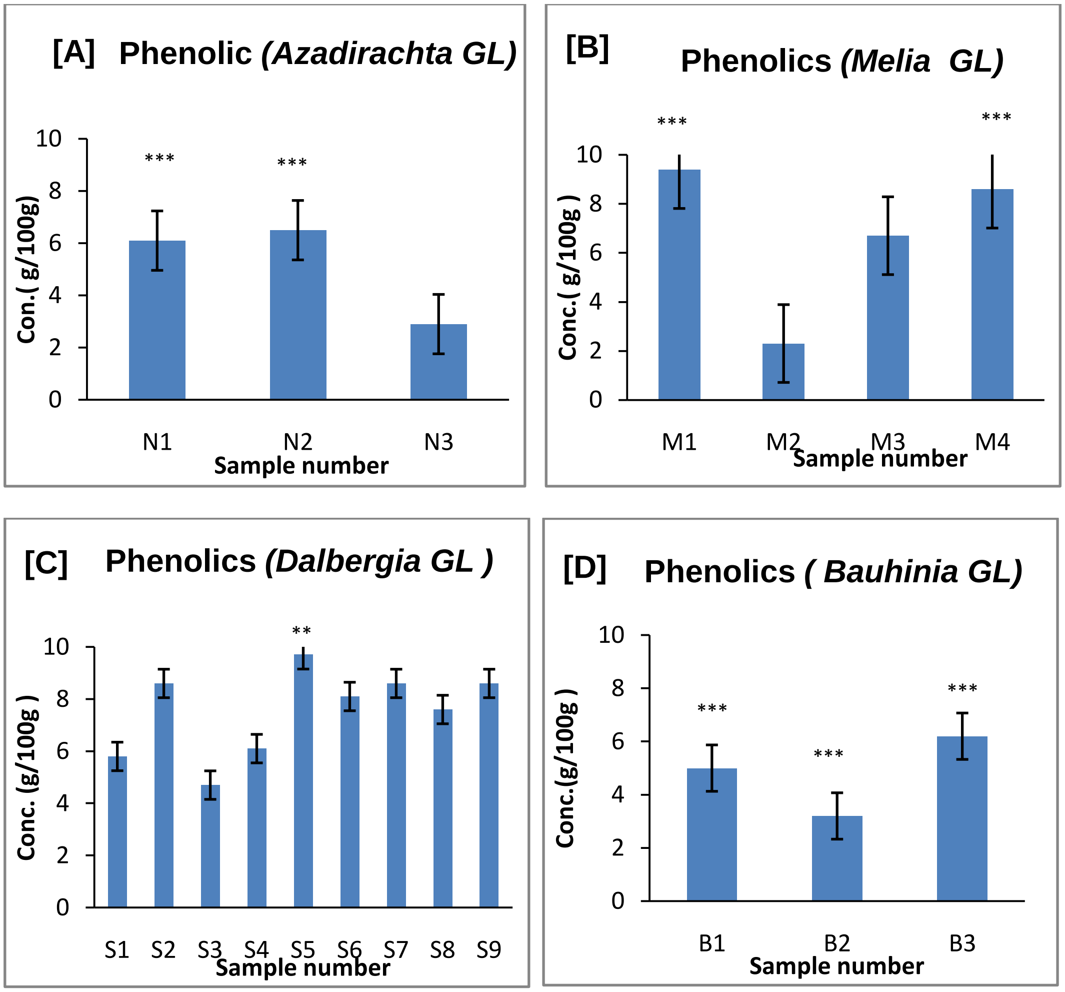
In Azadirachta GL, phenolic content was highest N1 (6.1g/100g) and N2 (6.5 g/100g) as compared to sample N3 which was in dried form. No significant difference was recorded between N1 and N2 ($P<0.001$), whereas N3 showed lowest phenolic content (2.9 g/100g), which is pertinent because of its dried stage (Fig 4.5A).

In Melia GL, M1 semi-dried form showed highest phenolic content of 9.4g/100g followed by M4 (8.6g/100g growing stage), which was significantly ($P<0.001$) lower than M1, followed by M3 and M2 (2.3 and 6.7g/100g)(Fig 4.5B).

Estimation of phenolic was done in nine samples of Dalbergia GL, in which S5, S2, S9 (9.7, 8.6, 8.6g/100g), which were in growing phase showed high content, whereas S1 (5.8), S4 (6.1g/100g) in dry form showed low phenolic content. Only sample, S5 showed significant differences in their mean values with S3 and S1 ($P<0.05$), whereas others did not show any difference in the phenolic content (Fig 4.5C).

In Bauhinia GL, highest content was recorded in B3 (6.2 g/100g) followed by B1 (5g) and B2 (3.2g/100g), which was significantly different ($P<0.001$) from each other. It was apparent from the results that the fruiting body fresh samples possessed highest phenolics content (Fig 4.5D).

In Acacia GL, highest phenolic content of 9.3g was observed in A8 (fresh fruiting body) followed by A4 (8.6g) and A3 (7.9g) respectively, all these samples were in growing stage and possessed significant differences ($P<0.001$) among them as compared to dried samples A1, A5 and A6 (Fig 4.5E).



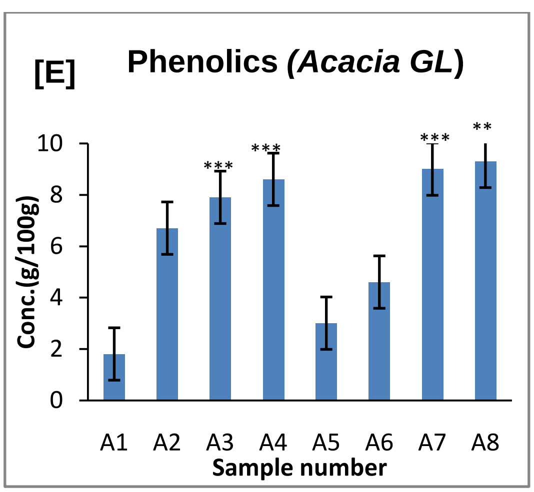


Figure 4.5 Phenolicscontent in [A] in Azadirachta GL [B] Melia GL, [C] Dalbergia GL, [D] Bauhinia GL, [E]Acacia GL.

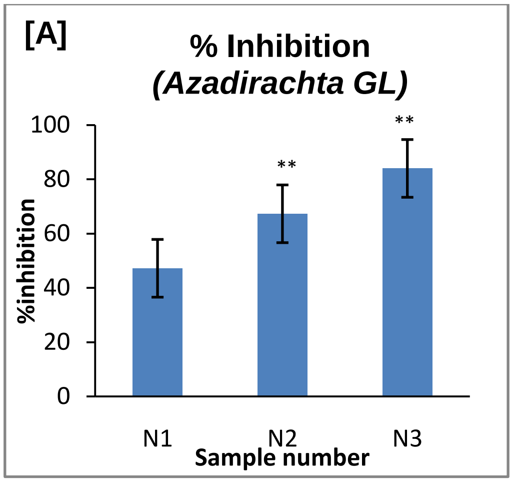
Level of significance was measured at

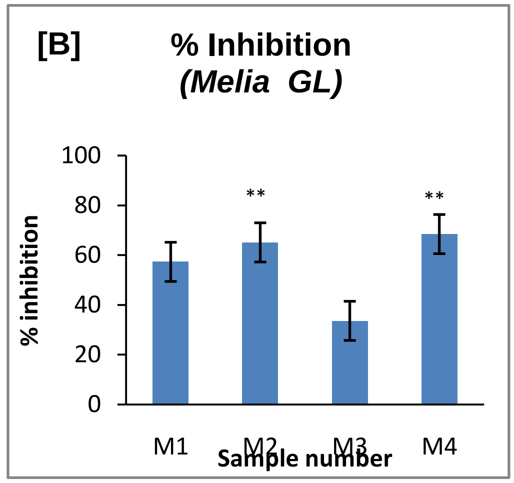
*** represent ($P<0.001$),

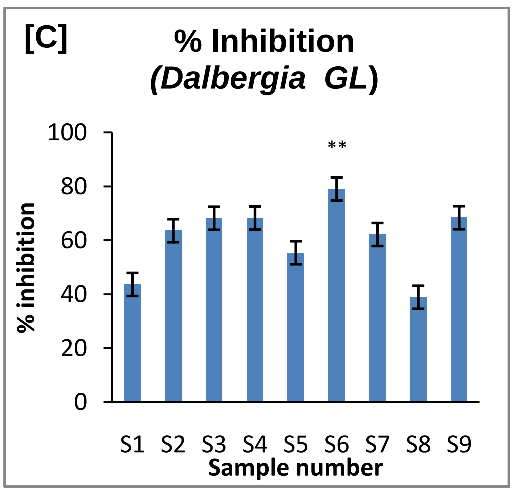
** represent ($P<0.005$)

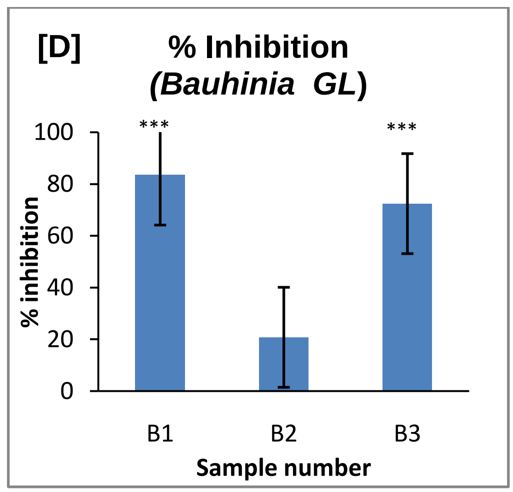
Similar phenolic content was seen in different host specific GL samples. M1 (9.4), S5 (9.7), A8 (9.3) g/100g showed almost similar phenolic content.

4.6 Antioxidant property in term of % inhibition:









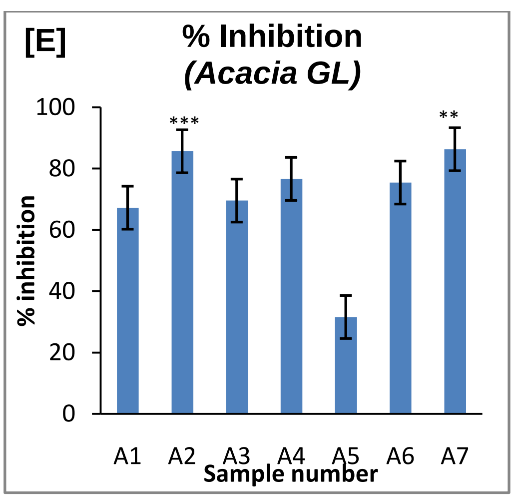


Figure 4.6 Antioxidant property in term of % inhibition [A] in Azadirachta GL [B] Melia GL, [C] Dalbergia GL, [D] Bauhinia GL, [E]Acacia GL.

Level of significance was measured at

*** represent (P<0.001),

** represent (P<0.005)

The percent inhibition was highest in N3 (83.98%) and N2 (67.26%) of Azadirachta GL as compared to N1 (47.1%). Significant differences were obtained within N1 vs. N2 and N1 vs. N3 ($P<0.05$) (Fig 4.6 A).

In case of Melia GL percent inhibition was highest in M2 (65.11) and M4 (68.44%) (values at par) but these were significantly higher than M1 (57.28 %), and M3 (33.56) ($P<0.05$) (Fig 4.6 B).

Dalbergia GL showed large variations in terms of % inhibition, highest percent inhibition was recorded in S6 (79%) followed by S9 (68.4%), S4 (68.3%), and S3 (68.2%). S6 had significantly higher ($P<0.001$) % inhibition than S9, S4 and S3. There were variations in the S9, S4 and S3 samples with significant differences ranging from $p<0.001$, 0.005 and 0.05 (Fig 4.6 C).

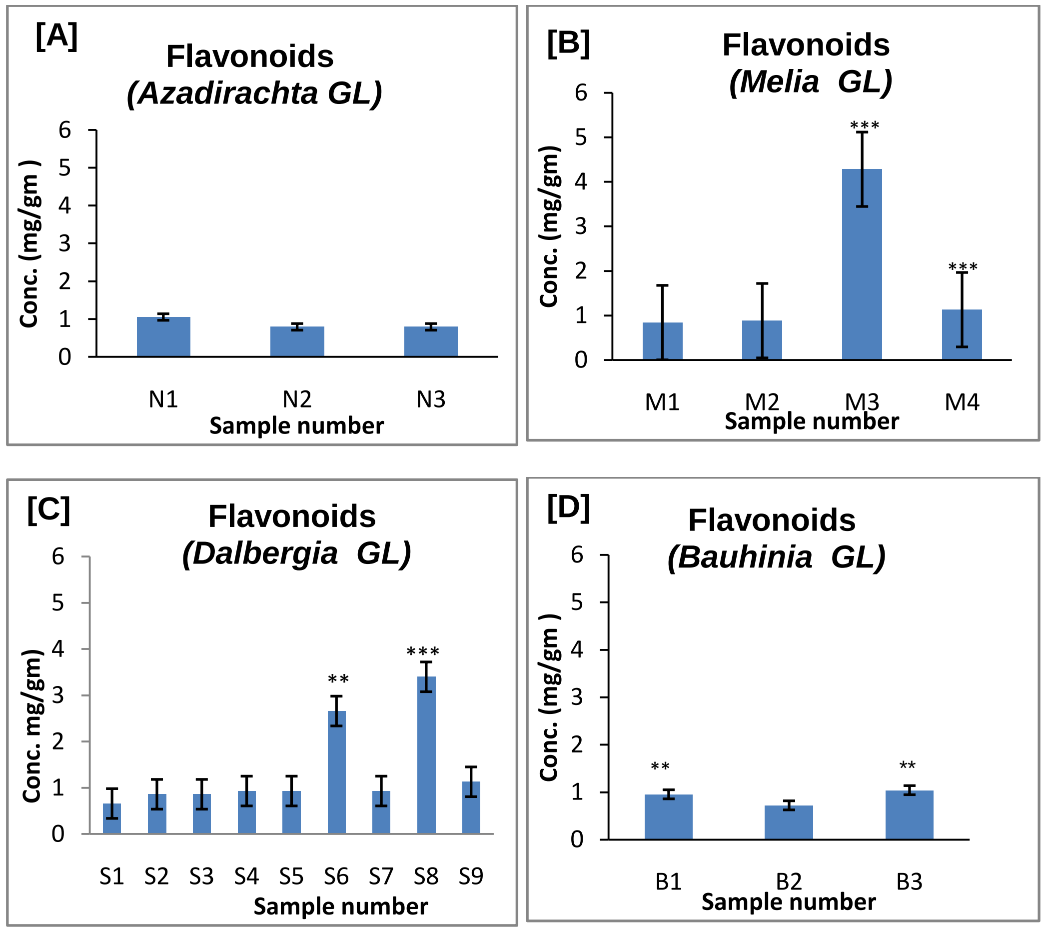
Large variations were observed in Bauhinia GL. Highest percent inhibition recorded was 83.45% in B1 followed by B3 (72.43%), which were significantly higher ($P<0.001$) than B2 (20%) ($P<0.001$) (Fig. 4.6 D).

In Acacia GL, overall inhibition percentage was quite higher than any other GL. Majority of the samples showed antioxidant property in terms of percent inhibition from 69%-86%. Significant differences ($P<0.05$) were obtained in different groups (A2 86%, A7 85%), (A4 76%, A6 75%) and (A1 69%, A3 67%) (Fig. 4.6 E).

4.7 Flavonoids content:

Flavonoids content was also estimated in Ganoderma lucidum samples collected from different host plants. In Azadirachta GL, there wasn’t any variation in the flavonoids content of N2 (0.8 mg/g) and N3 (0.8mg/g) but in N1 (1.06 mg/g) the flavonoids content was significantly higher than N2 and N3 ($p<0.05$) (Fig 4.7A).

In Melia GL, M3 showed highest flavonoid content (4.2mg/g), which was exceptionally higher ($P<0.001$) than M4 (1.13), M2 (0.88) and M1 (0.84) mg/g (Fig 4.7B). In Dalbergia GL, S8 showed highest flavonoid content (3.4mg/g) which was significantly higher than S6 (2.66mg/g) and remaining samples. Though there were significant differences within the remaining samples but the content was 4-5 folds less than the S8 (Fig 4.7C). Azadirachta GL and Bauhinia GL did not show any kind of variation in flavonoid content with respect to their stage. Melia GL, Dalbergia GL and Acacia GL had highest flavonoid content with respect to the growing stage while it was low in dried samples. Highest flavonoid content was seen in Acacia GL A3 (4.26), A8 (5.26) mg/g while in Dalbergia GL sample possessed lowest S1 (0.6 mg/g) content (Fig 4.7D, E).



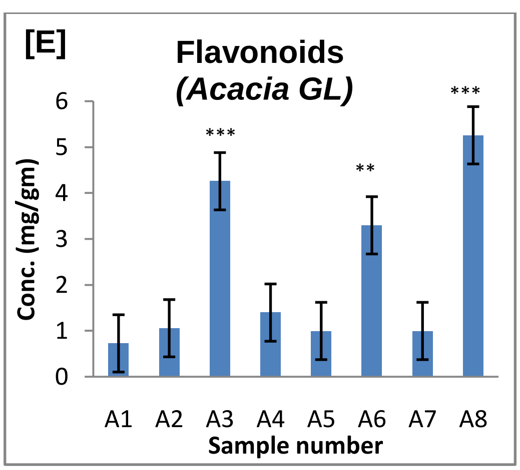


Figure 4.7 Flavonoids content different host specific samples of GL [A] in Azadirachta GL [B] Melia GL, [C] Dalbergia GL, [D] Bauhinia GL, [E]Acacia GL.

Level of significance was measured at

*** represent (P<0.001),

** represent (P<0.005)

4.8 Terpenoids profiling using Thin Layer Chromatography:

Different spots were observed on TLC plates representing different terpenoids. Each spot may represent more than one compound, which can interact in the same way with the stationary and the mobile phase. A total of sixteen R_f values were seen as single spots or clusters in the TLC of terpenoids in different samples obtained from different hosts (Denoted as T1-T16) in (Table 4.1). T1 was common in Bauhinia GL, Melia GL, Azadirachta GL and Dalbergia GL but was absent in Acacia GL. T2 and T3 were only visible in Azadirachta GL. T4 was only available in Melia GL, T5 and T5 were only seen in Acacia GL, T7 in Melia GL,T8 in Bauhinia GL and Dalbergia GL, T9 in Dalbergia GL only, T10 in Acacia GL only, T11 in Bauhinia GL only T12 in Melia GL and Azadirachta GL, T13 in Acacia GL and Melia GL only, T14 in Melia GL only, T15 in Dalbergia GL only and T16 in Bauhinia GL only (Table 4.1). The TLC analysis showed variations in the terpenoids among various hosts specific GL samples. The single spot were more prominent in Azadirachta, Melia, Acacia, signifying unique terpenoids.

Table 4.1: R_f values of terpenoids of GL

Terpenoids	***Acacia GL***	***Bauhinia GL***	***Melia GL***	***Azadirachta GL***	***Dalbergia***	***Common and individual terpenoids***
T1		0.18	0.19	0.19	0.18	Bauhinia GL, Melia GL Azadirachta GL, Dalbergia GL
T2				0.23		Azadirachta GL
T3				0.33		Azadirachta GL
T4			0.54			Melia GL
T5	0.64					Acacia GL
T6	0.66					Acacia GL
T7			0.68			Melia GL
T8		0.70			0.70	Bauhinia GL, Dalbergia GL
T9					0.71	Dalbergia GL
T10	0.74					Acacia GL
T11		0.75				Bauhinia GL
T12			0.77	0.77		Melia GL, Azadirachta GL
T13	0.81		0.81			Acacia GL, Melia GL
T14			0.86			Melia GL
T15					0.88	Dalbergia GL
T16		0.90				Bauhinia GL

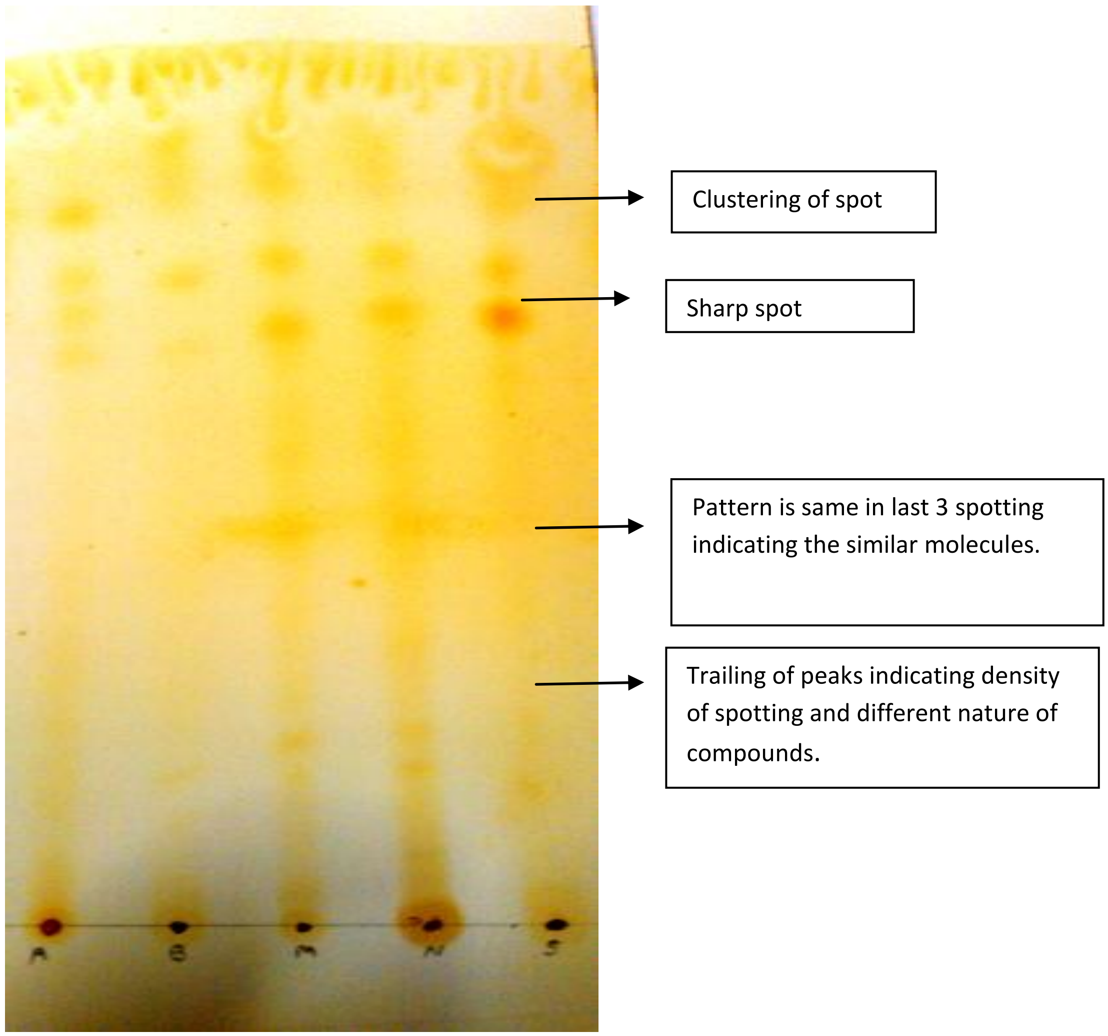


Figure 4.8(A): Terpenoids profiling using Thin Layer Chromatography (TLC)

Solvent system = Chloroform: Methanol (10:1)

A = Acacia

B = Bauhinia

M = Melia

N = Azadirachta

S = Dalbergia

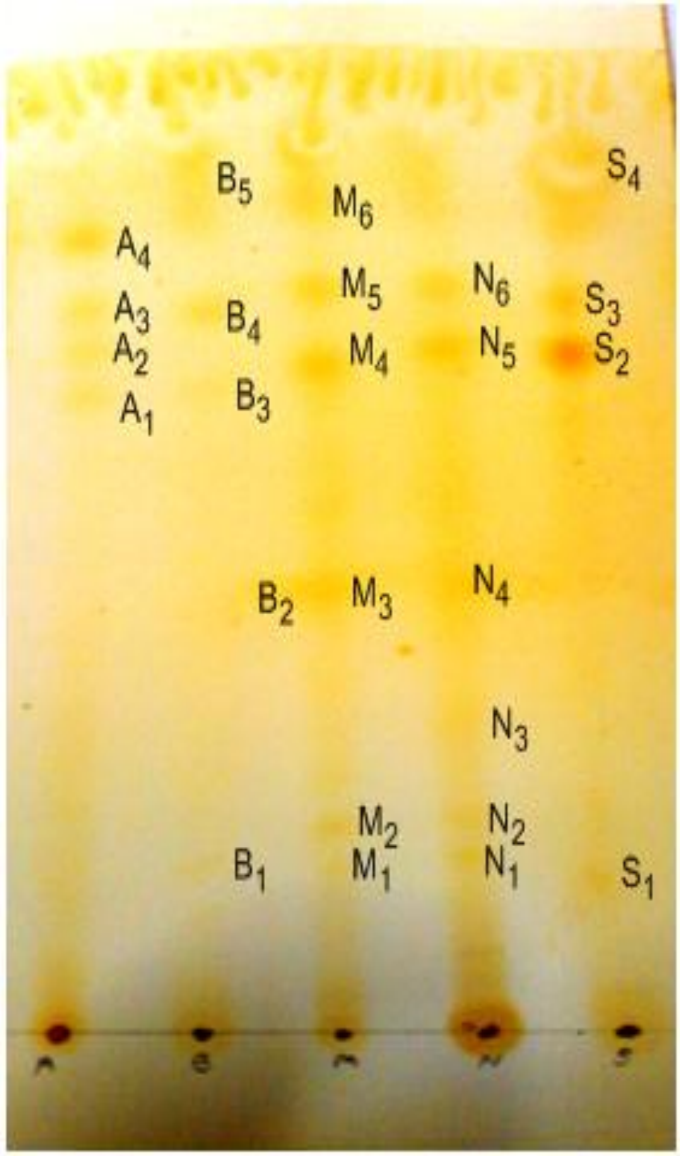


Figure 4.8(B): Terpenoids profiling using Thin Layer Chromatography (TLC)

4.9 Polysaccharides analysis using TLC:

The pooled samples were also processed for the extraction of polysaccharides by the method of Aryantha et.al 2002. TLC pattern showed the presence and abundance of polysaccharides in G. lucidum. The preliminary data in the form of R_f value described the abundance of polysaccharides in all the samples of Ganoderma and further elucidation of particular polysaccharide is required.

Table 4.2: R_f values of polysaccharides of GL

Name of the host	**Rf value**
Acacia GL	0.53
Bauhinia GL	0.52
Melia GL	0.53
Azadirachta GL	0.54
Dalbergia GL	0.51

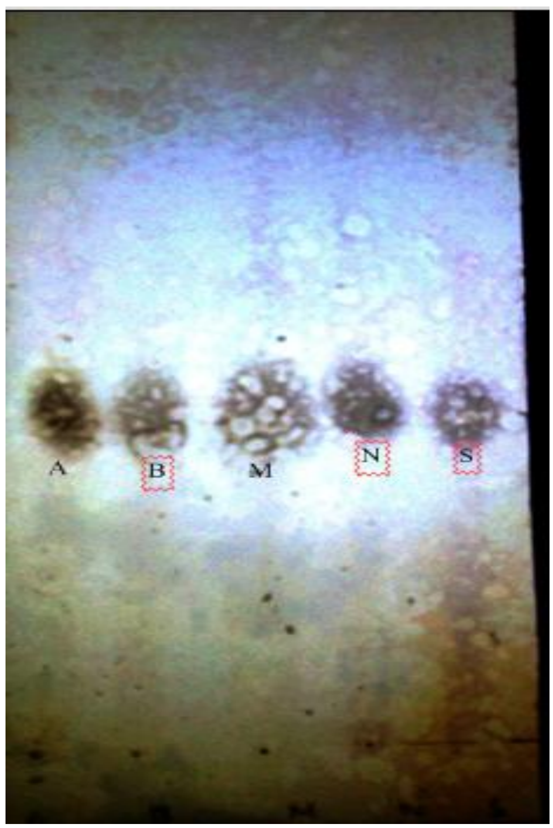


Figure 4.9. Polysaccharides profiling using Thin Layer Chromatography (TLC).

A = Acacia, B = Bauhinia, M = Melia, N = Azadirachta, S = Dalbergia

4.9 High Pressure Liquid Chromatography (HPLC) for Ganoderic acid: Finally the different Ganoderma samples were subjected to UHPLC. Ganoderic acid has weak solubility in aqueous media due to high hydrophobicity. Due to high solubility and good eluting effect of Ganoderic acid, methanol mixed with water was selected as mobile phase and absorbance was recorded at 245 nm with a run time of 20 minutes. In HPLC, different peaks were obtained at different retention time; some were common in all Ganoderma samples, whereas some were only seen in particular host specific fungus. At retention time of 1.6 only Dalbergia GL showed peak, whereas at 2.8 only Melia GL showed peak, but at RT of 3.6 and 4.1 all the Ganoderma samples showed peaks. Only Melia GL showed peaks at RT 4.4, 5.6 and 5.8. Acacia GL, Azadirachta GL and Dalbergia GL showed single peak at RT 6.1. At RT 6.3 only Melia GL showed the peak. At RT 6.6 only Acacia GL showed the peak but at 7.2 four GL samples (Acacia GL, Melia GL, Azadirachta GL and Dalbergia GL) showed common peak. Melia GL and Azadirachta GL had common peaks at 8.8. Only Azadirachta GL showed peaks at 9.6 and 11.1. It can be concluded that there is variability of GA isoforms in different host samples. If we exclude the common peaks, single peak represents a chemo-variant based on the GA. Melia GL showed maximum variability among other GL samples. Further confirmation is required for exact elucidation of the chemo-variants on the basis of GA.

Table 4.3: Data regarding peaks of different samples of GL in HPLC

Retention time	***Acacia GL***	***Bauhinia GL***	***Melia GL***	***Azadirachta GL***	***Dalbergia GL***
1.6					✓
2.8			✓		
3.6	✓	✓	✓	✓	✓
4.1	✓	✓	✓	✓	✓
4.4			✓		
4.6	✓	✓			
4.8			✓		
5.6			✓		
5.8			✓		
6.1	✓			✓	✓
6.3			✓		
6.6	✓				
7.2	✓		✓	✓	✓
8.8			✓	✓	
9.6				✓	
11.1				✓	

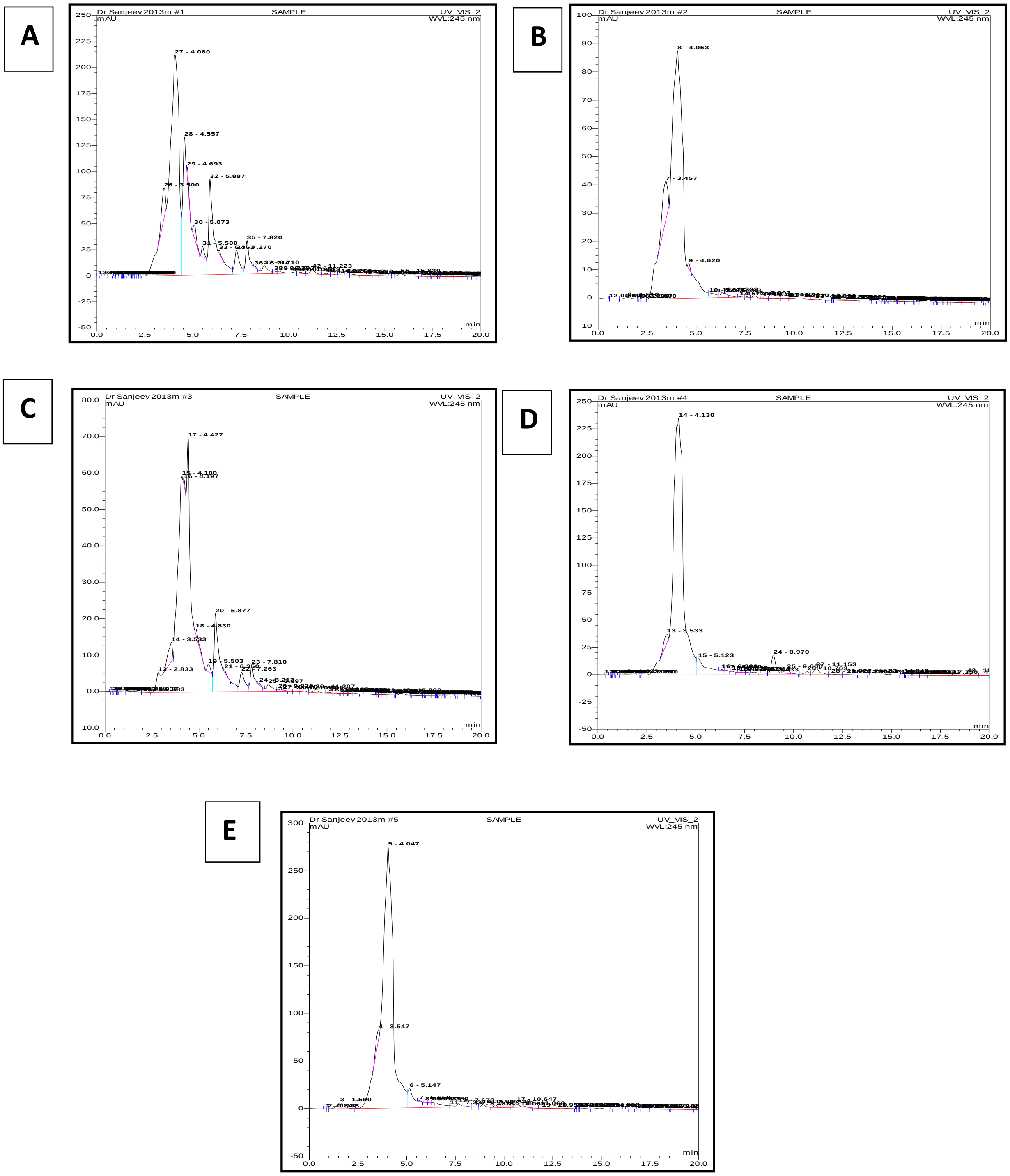


Figure 4.10 Chromatogram of GA in different hosts **[A]** Acacia, **[B]** Bauhinia**,** **[C]** Melia, **[D]** Azadirachta, **[E]** Dalbergia

Accordance or conflict among the results is presented in the next chapter.

CHAPTER V

DISCUSSION

Ganoderma lucidum is generally seen in different parts of India. A major portion of it is found in Western Ghats and adjoining areas. It grows in those areas where conditions are favorable and nutrients are easily available. Rainfall and temperature are important factors for its growth. Since this fungus is rich in different secondary metabolites, variation in production of different secondary metabolites depends upon the temperature, humidity and rainfall. Production of terpenes is negatively correlated with rainfall, in fact some of the terpenes found only in summer season (Vallat et al., 2005). Bathinda is a semi-desert location, where rainfall is scanty and temperature shows lots of variation (-2° to 48°C) still this fungus is in abundance. In this kind of climate, Ganoderma lucidum need some special kind of system to survive. It may vary or alter composition of secondary metabolite to combat with harsh conditions of Bathinda.

The chemical profiling of Ganoderma lucidum was carried out to understand the influence of stressed environment on the chemical composition of host specific fungus. It has been well elucidated that Ganoderma lucidum consists of ganoderic acid, polysaccharides, proteins, which act as key anti-cancer molecules as evident from literature (Xu et al., 2011). The host specific samples were collected and subjected to different parameters such as total sugars, reducing sugars, starch, proteins, phenols, anti-oxidant property and flavonoids, terpenoids and ganoderic acid profile.

The present study revealed that the sugar/ carbohydrate content in the Bathinda samples is 1.4 to 3.3 folds higher than the available reports from Himalayan region (Rawat et al.) and China (Mau et al., 2001). Carbohydrate content has been reported 27g/100g and 20.21g/100g dry weight in the Himalayan and China's GL, but in the present study 5.07- 38.1g/100g carbohydrate content was recorded in Acacia GL, this variation in the samples of Acacia GL is due to condition of the samples.The variation in the total sugar and reducing sugar can be attributed to the different hosts and adverse climatic conditions of Bathinda and its adjoining region. There are strong evidences regarding the influenceof abiotic stress signals on secondary metabolites.

They often occur in plants in response to various stresses (Ramakrishna and Ravishankar, 2011).

The overall content of carbohydrate is high in Azadirachta GL. Elevated content of total sugars is an indication that there may be possibility of pharmacologically active compounds i.e. sugar, sugar alcohol etc.

In the fresh samples (fruiting bodies) of Azadirachta GL, the reducing sugars were 15.9 fold higher as compared to 1.19±0.14% of GL of Uttarakhand (Rawat et al. 2012).

The starch content was exceptionally higher in our samples (57.3g/100g), which were1.9 folds higher than the polysaccharides content of Uttarakhand Ganoderma lucidum (29.25g/100g) (Rawat et al.2012).

It is very well established that Ganoderma active ingredients included high molecular weight polysaccharides, triterpenoids, organic germanium adenosine etc. (Chang, 1996). Majority of these active molecules have therapeutic effects which consist of mainly antitumor activity, immunomodulating and chronic bronchitis (Wasser et al., 1999). Ganoderma available in Bathinda has much higher concentration of sugars and starch.

Phenolic compounds and ascorbic acid are known to have antioxidant properties. In present case, phenolic content was also observed higher in our samples as compare to the available reports on Ganoderma tsugae; 47 mg/g (Mau et al., 2002), in G. boninene, it was 25.02 mg/g (Mitchell et al., 2009), in south Indian Ganoderma lucidum, it was 42.42 mg/g (Rajasekaran et al., 2011). It ranged from 1.8 -9.7g/100g, which is almost double than any other GL (18-97mg/g). It was almost 2.06 fold higher than G. tsugae, 3.8 fold higher than G. boninene and 2.2 fold higher than South Indian Ganoderma lucidum. Large variation in the phenolic content of our GL samples is mainly due the sample condition i.e. fresh vs. dried, also the doubling of the content may be due altered metabolic constituents required to cope up with harsh climate and differences in the host plant.

Generally, mushrooms are rich in the proteins but the medicinal mushrooms does not possess high protein content (Crisan et al., 1978). In present case, it was highest in Dalbergia GL (42g/100g), at the same time found lowest in Bauhinia GL

(4.2g/100g). Apparently, the content was 2.03 fold higher than the Himalayan Ganoderma lucidum (20.62 g/100g) (Rawat et al.2012). The higher protein content in this mushroom is an advantage in terms of its further exploration in the field of pharmacology. No such work is reported so far and best of our knowledge.

The free radical scavenging effect on DPPH was measured in terms of % inhibition, which was 86.31% in Acacia GL followed by 83.98% in Azadirachta GL. The antioxidant property of Punjab Ganoderma lucidum samples was much higher (1.6 -1.19 folds) than already reported (Mau et al., 2002)51.2 and 52.6%, 70%(Kim et al., 2008) and 72.24% (Rajasekaran and Kalaimagal, 2011) in South Indian GL samples. In our case, high phenolic content and anti-oxidative property can be correlated with the fact that in South West Punjab the fungus is growing on different hosts and adverse climatic conditions. Accumulation of phenolics is the result of defense against harsh conditions.

In present study, the flavonoids content was 5.26 mg/g in Acacia GL which is relatively low as compared to G. boninene (16.89 mg/g) (Mitchell et al., 2009) and South Indian Ganoderma lucidum (13.57 mg/g) (Rajasekaran and Kalaimagal, 2011). A precise composition of biomolecules is given in the table mentioned below:

Table 5.1: Biochemical constituents in g/100g of the dry weight of GL

S. No	**Biochemical constituents**	**% composition**
1	Total sugars	38.1± 0.0481%
2	Reducing sugars	19± 5.925%
3	Starch	57.3± 3.33%
4	Proteins	42± 4.2%
5	Phenols	9.7± 0.066%
6	Scavenging %(DPPH assay)	86.31± 5.48%
7	Flavonoids	0.52±0.6%

Where total sugars are 38.1%, starch is 57.3%, proteins are 42%, phenols are 9.7% and flavonoids are 0.52% in g/100g of dry weight.

The TLC and HPLC analysis confirmed the availability of two major classes of bioactive ingredients; polysaccharides and lanostane type terpenoids mainly ganoderic acids, ganoderic alcohols and their derivatives (Gao and Zhou, 2003). TLC of terpenoids showed cluster as well as individual spots indicating abundance of terpenoids and also variation in the host specific Ganoderma. Polysaccharides are also in abundance in these samples which givesenough scope for further characterization of individual active molecule mainly glycan's and glycoproteins. The ganoderic acid is also available in our samples and there are variations in the peaks at different retention time. Individual peak represents a different isoforms of ganoderic acid and characterize the Ganoderma as chemo-variant. The ganoderic acid samples are being analyzed further for individual ganoderic acid isoforms.

SUMMARY AND CONCLUSION

It can be summarized from the preliminary data collected from the present study that different biomolecules are present within the different host to different extent. The content of the active biomolecules ranges from few to several folds higher than the available reports, especially the variation in the terpenoids and ganoderic acid which was very much apparent. The results obtained, as a consequence of the research carried out, is summarized as follows:

Carbohydrate:

6.1 Total sugars: Carbohydrates are important source of energy, providing energy through metabolic pathways. It supplies carbon for the synthesis of various secondary compounds. Total sugars content was observed to be maximum in Acacia GL 38.1g/100g. In general following trend was observed host wise:

Acacia GL >Azadirachta GL>Dalbergia GL>Melia GL>Bauhinia GL

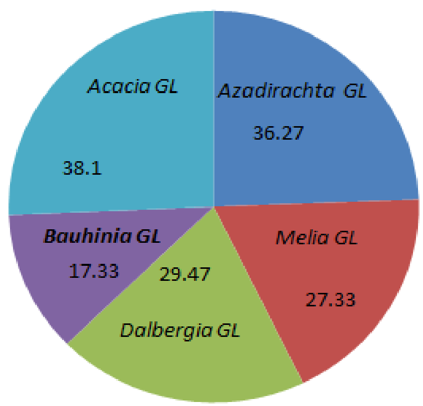


Figure 6.1 Total sugars content (g/100g) in different host specific samples of GL

6.1.1 Reducing sugars: Reducing sugars were highest in Azadirachta GL; 19 g/100g. Following Trend was observed in total sugars content:

Azadirachta GL>Melia GL>Acacia GL>Bauhinia GL >Dalbergia GL

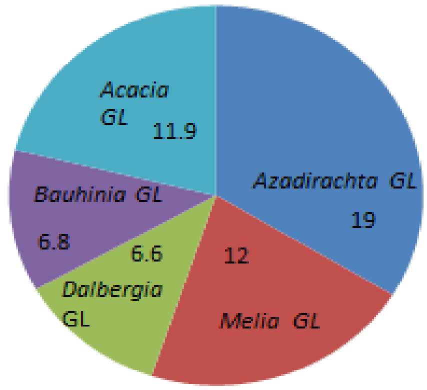


Figure 6.1.1 Reducing sugars content (g/100g) in different host specific samples of GL.

6.1.2 Starch: starch content was maximum 57.3 g/100g in Acacia GL and 41 g/100g in Azadirachta GL. Starch content in host specific GL was in following manner

Acacia GL >Azadirachta GL>Dalbergia GL >Melia GL >Bauhinia GL

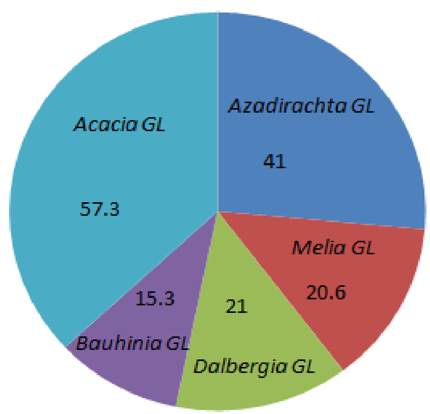


Figure 6.1.2 Starch content (g/100g) in different host specific samples of GL

6.2 Proteins: The protein content in our samples was much higher as compared to other mushrooms. Maximum proteins content in our samples was seen in Dalbergia GL (42g/100g) which was much higher than already reported Himalayan samples. Following trend was seen in the protein content.

Dalbergia GL >Melia GL>Azadirachta GL>Acacia GL > Bauhinia GL

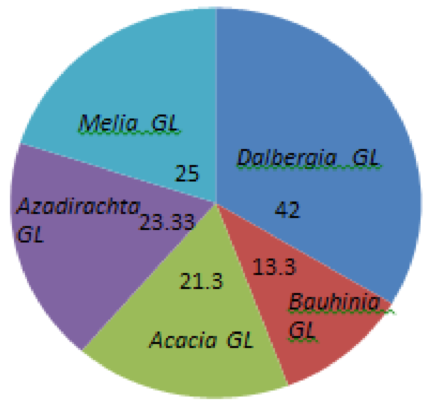


Figure 6.2 Proteins content (g/100g) in different host specific samples of GL

6.3 Phenolic Content: The highest phenolic content of 97mg/g was observed at the fruiting stage of Dalbergia GL while the lowest content was seen in Acacia GL. Higher phenol content is directly proportional to the adaptation to harsh climate. Phenylpropanoids are increased in extreme condition enabling the host to survive.Different health benefits of phenols make it a valuable mushroom**.**

Dalbergia GL>Melia GL>Acacia GL>Azadirachta GL>Bauhinia GL

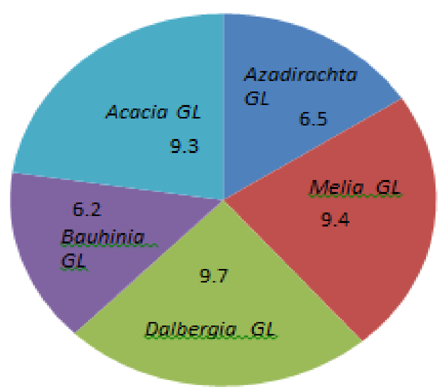


Figure 6.3 Phenolics content (g/100g) in different host specific samples of GL

6.4 Antioxidant property: Free radical scavenging capacity in terms of % inhibition was found to be highest in Acacia GL (86.38%) and Azadirachta GL (83.98%), which was exceptionally higher than in any other report. The overall content was as below:

Acacia GL >Azadirachta GL>Bauhinia GL >Dalbergia GL >Melia GL

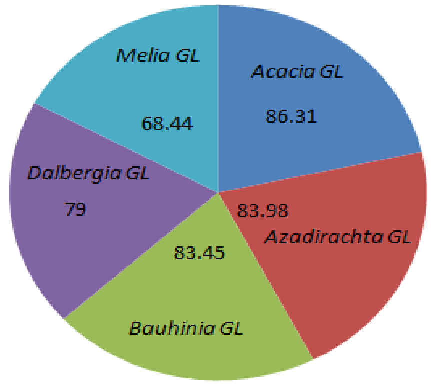


Figure 6.4 Antioxidant property in term of % inhibition in host specific samples of GL

6.5 Flavonoids: Flavonoids content was not high in our samples with a maximum content recorded as 5.26 mg/g in Acacia GL. Nevertheless, the antioxidant activity was found to be much higher in our samples, which can be associated with high phenolic content and flavonoids.

Acacia GL >Melia GL >Dalbergia GL >Azadirachta GL > Bauhinia GL.

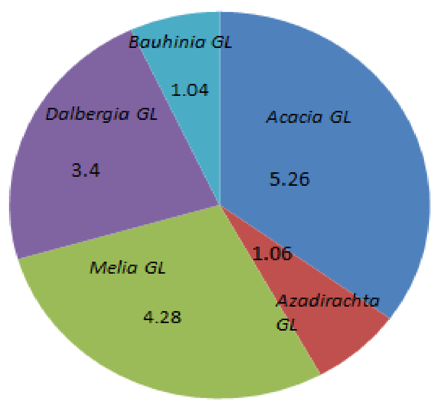


Figure 6.5 Flavonoids content (mg/100mg) in different host specific samples of GL.

6.6 Terpenoids and ganoderic acid profile:

The TLC analysis for terpenoids showed variations and abundance among the Ganoderma samples. GA analysis also showed presence and availability of ganoderic acid (GA). Due to unavailability of individual GA standards further quantification could not be completed.

Conclusion:

Ganoderma lucidum is an important medicinal mushroom possessing effective therapeutic activity against a variety of human diseases. Amount of biomolecules present in the body set up the functioning of the body and decides growth and development. In this study the amount of total sugars present in the Ganoderma samples was observed to be 38.1g/100g, starch content was 57.3g/100g and protein content was 42g/100g, which is much higher than the available reports thus, making it a valuable herb and theurapeutic repository for the treatment of various diseases. High free radical scavenging property in terms of DPPH inhibition percentage (86.31%) designates it as a strong antioxidant. High phenolic content (97mg/g) supports its antioxidant property. The TLC pattern opens a way to explore the difference in the availability of terpenoids with in different host.

G. lucidum is a valuable medicinal mushroom, a boon given by nature to human being. There are variations in the composition of different biomolecules in host specific Ganoderma samples; therefore every host is giving a chemo-variant. This would be quite useful in testing of this Ganoderma lucidum extract (GLE) and Ganoderma lucidum Polysaccharides (GLP) against cancer and other diseases. Further research is in progress for estimation of individual ganoderic acid and their role as potent anticancer molecule.

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